MPMI Vol. 36, No. 12, 2023, pp. 764–773, https://doi.org/10.1094/MPMI-05-23-0067-IRW

INTERACTIONS REVIEW

The Necrotrophic Pathogen *Parastagonospora nodorum* Is a Master Manipulator of Wheat Defense

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Accepted for publication 11 August 2023.

***Parastagonospora nodorum* is a necrotrophic pathogen of wheat that is particularly destructive in major wheat-growing regions of the United States, northern Europe, Australia, and South America. *P. nodorum* secretes necrotrophic effectors that target wheat susceptibility genes to induce programmed cell death (PCD), resulting in increased colonization of host tissue and, ultimately, sporulation to complete its pathogenic life cycle. Intensive research over the last two decades has led to the functional characterization of five proteinaceous necrotrophic effectors, *SnTox1*, *SnToxA*, *SnTox267*, *SnTox3*, and *SnTox5*, and three wheat susceptibility genes, *Tsn1*, *Snn1*, and *Snn3D-1*. Functional characterization has revealed that these effectors, in addition to inducing PCD, have additional roles in pathogenesis, including chitin binding that results in protection from wheat chitinases, blocking defense response signaling, and facilitating plant colonization. There are still large gaps in our understanding of how this necrotrophic pathogen is successfully manipulating wheat defense to complete its life cycle. This review summarizes our current knowledge, identifies knowledge gaps, and provides a summary of well-developed tools and resources currently available to study the *P. nodorum*–wheat interaction, which has become a model for necrotrophic specialist interactions. Further functional characterization of the effectors involved in this interaction and work toward a complete understanding of how *P. nodorum* manipulates wheat defense will provide fundamental knowledge about this and other necrotrophic interactions. Additionally, a broader understanding of this interaction will contribute to the successful management of Septoria nodorum blotch disease on wheat.**

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Funding: This work was supported by the U.S. Department of Agriculture, Agricultural Research Service through CRIS project 3060-22000-051-00D.

e-Xtra: Supplementary material is available online.

The author(s) declare no conflict of interest.

Keywords: effector, fungal pathogen, necrotrophic effector, *Parastagonospora nodorum*, wheat

Background

Much is known about how biotrophic pathogens infect their respective hosts, including the activation of defense pathways via the recognition of pathogen-associated molecular patterns (PAMPs) that results in PAMP-triggered immunity (PTI) and the recognition of pathogen-produced effectors by various intracellular receptors that leads to effector-triggered immunity (ETI) (Chisholm et al. 2006; Dodds and Rathjen 2010; Jones and Dangl 2006). Additionally, findings have shown that PTI is required for complete activation of ETI and that the components of ETI are required for the stabilization of PTI (Bjornson and Zipfel 2021; Ngou et al. 2021; Yuan et al. 2021). Therefore, PTI and ETI work in tandem to provide resistance against many plant pathogens. Necrotrophic pathogens, however, often target PTI or ETI pathways by using necrotrophic effectors to trigger programmed cell death (PCD) (Faris et al. 2010; Lorang et al. 2007; Shi et al. 2016), resulting in necrotrophic effector-triggered susceptibility (Faris and Friesen 2020; Friesen and Faris 2021; Liu et al. 2012). *Parastagonospora nodorum* (Berk.) Quaedvlieg, Verkley & Crous. (Quaedvlieg et al. 2013), the causal agent of Septoria nodorum blotch (SNB) is a necrotrophic specialist fungal pathogen of wheat and is a problem in the northern Great Plains and southeastern regions of the United States, northern Europe, northern India, parts of Brazil, and Western Australia (Bhathal et al. 2003; Downie et al. 2021; Katoch et al. 2019; Mehra et al. 2019). Under favorable conditions, the fungus produces and releases ascospores or conidia that infect wheat leaves. The pathogen releases necrotrophic effectors that manipulate the host immune system and activate PCD, producing necrotic lesions in susceptible wheat genotypes with the corresponding host susceptibility genes (Friesen and Faris 2010). *P. nodorum* thrives on necrotic material to acquire nutrients to complete its life cycle (Fig. 1). Because the necrotrophic effector–susceptibility gene interaction results in susceptibility, the *P. nodorum*–wheat interaction follows an inverse gene-for-gene model (Friesen and Faris 2010), which is a mirror image of the classical gene-for-gene model (Flor 1954) that is typical of plant–biotrophic pathogen interactions.

To date, six proteinaceous necrotrophic effectors have been identified in *P. nodorum*, five of which have been cloned and functionally characterized to different levels (Friesen and Faris 2021). The objective of this review is to highlight the progress and our understanding of the *P. nodorum*–wheat interaction,

focusing primarily on the cloning, functional characterization, and modes of action of known necrotrophic effectors and their host targets. Additionally, we will identify the gaps in our understanding of this interaction, propose future research to fill those gaps, and provide a summary of genetic and genomic resources that are available for studying this interaction.

Current Knowledge

P. nodorum was long thought to rely on cell wall-degrading enzymes to cause disease. Work in the last two decades has shown that, in addition to classical virulence effectors, *P. nodorum* produces necrotrophic effectors that target vulnerable host susceptibility genes/pathways to colonize and complete its pathogenic life cycle.

SnTox1 induces PCD and protects the pathogen from wheat chitinases

SnTox1 is a small, secreted protein that induces light-dependent PCD in wheat genotypes harboring the dominant susceptibility gene, *Snn1* (Liu et al. 2004, 2012; Shi et al. 2016). The SnTox1-*Snn1* interaction was the first necrotrophic effector–susceptibility gene interaction identified in the *P. nodorum*–wheat system, and it contributed significantly to SNB disease at the seedling stage in the greenhouse (Liu et al. 2004) and at the adult plant stage in the field (Lin et al. 2022; Phan et al. 2016)

SnTox1 was cloned using a candidate effector list prioritized using known characteristics of effectors (Liu et al. 2012). *SnTox1*, which was located on *P. nodorum* chromosome 6 (Kariyawasam and Friesen 2021), encoded a 117-amino acid (aa) protein that consisted of a 17-aa signal peptide and a 100-aa mature protein (Liu et al. 2012), with 16 of the 100 aa being cysteines, making SnTox1 one of the most cysteine-rich proteins in the *P. nodorum* proteome (Friesen and Faris 2021). The prediction of eight disulfide bridges indicated a protein that had evolved to be stable in the harsh apoplastic environment. Subsequent protein localization using both a Tox1- green fluorescent protein (GFP) fusion and immunolocalization showed that this protein was indeed localized to the apoplast (Liu et al. 2016; Fig. 2; Supplementary Fig. S1).

Expression of *SnTox1* was detected as early as 3 hours postinoculation (hpi) and peaked at 72 hpi, coinciding with the

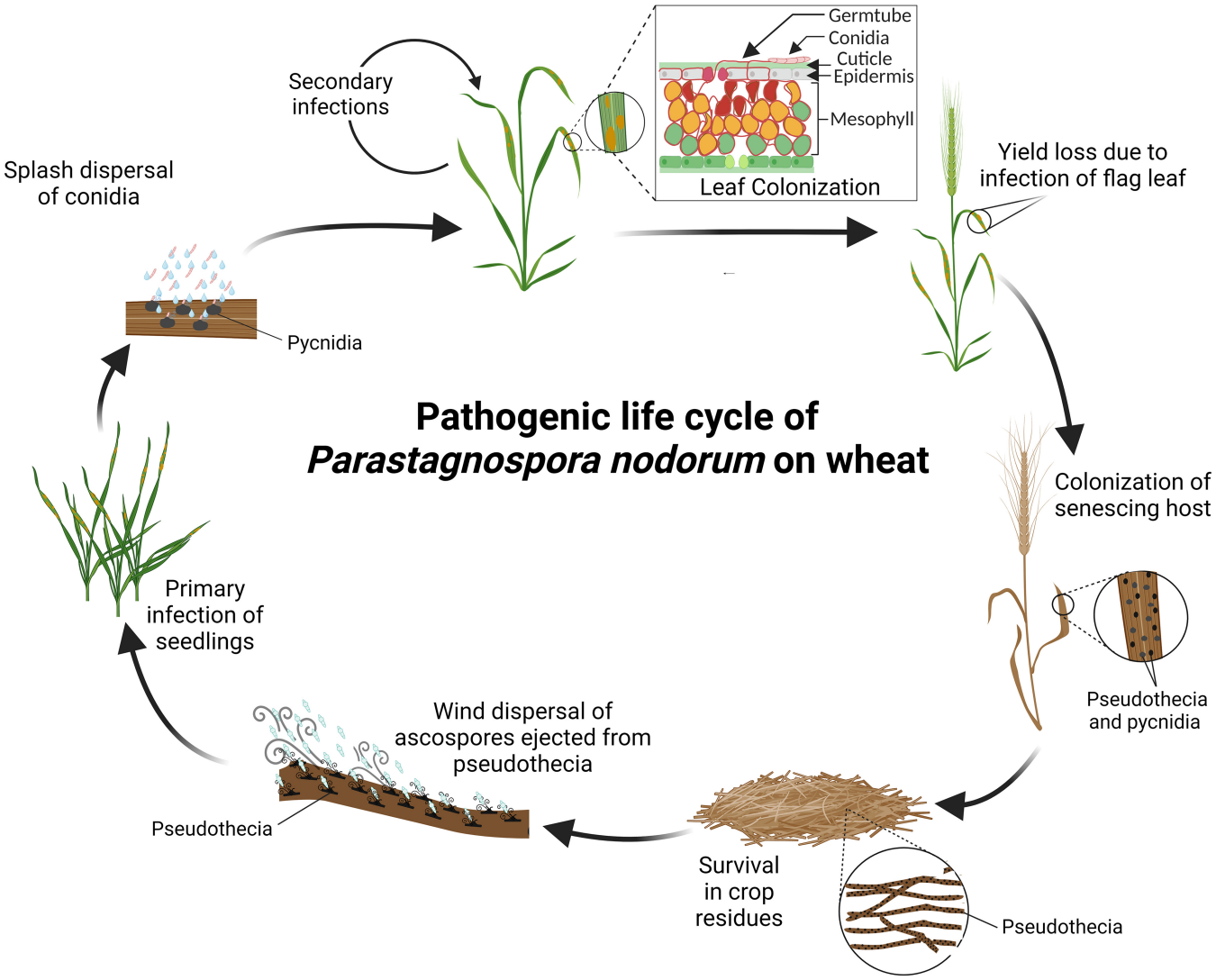

Fig. 1. Pathogenic life cycle of *Parastagonospora nodorum* on wheat. *P. nodorum* overwinters as pseudothecia in crop residue. Under wet and warm conditions, these pseudothecia release ascospores at the beginning of the growing season. Ascospores infect wheat seedlings, producing necrotic lesions. Pycnidia that develop in these lesions release conidia that are splash dispersed up the wheat plant, causing secondary infection cycles on wheat throughout the growing season. Infection on the flag leaf leads to the most yield loss. Complete colonization of the senescing host leaves and stems leads to the formation of pycnidia and eventually pseudothecia that are effective as overwintering structures. Created with www.BioRender.com.

onset of visible necrotic lesions (Liu et al. 2012). The SnTox1-Snn1 interaction triggered an oxidative burst, upregulation of *PR* genes such as *PR1-A1*, chitinases, and thaumatin-like protein genes, DNA laddering, and PCD that led to the formation of necrotic lesions, all of which are hallmarks of the host-controlled defense response against biotrophic pathogens (Supplementary Fig. S1). Therefore, it was hypothesized that SnTox1 was likely manipulating the host immune system to induce PCD.

Shi et al. (2016) showed that *Snn1* encoded for a wall associated kinase (WAK) that served as a pattern recognition receptor (PRR) (Fig. 2; Supplementary Fig. S1). WAKs that act as PRRs in other systems have been shown to recognize damage-associated molecular patterns to trigger host defense (Brutus et al. 2010). Furthermore, upon direct interaction between SnTox1 and Snn1, Shi et al. (2016) showed strong upregulation of *TaMAPK3* within 15 min of inoculation, which was typical of PTI, suggesting that SnTox1, in fact, was manipulating PTI to induce PCD to the advantage of the pathogen.

Population genetics studies showed that *SnTox1* was present in 84 to 95% of isolates in various global populations of *P. nodorum* (Liu et al. 2012; McDonald et al. 2013; Richards et al. 2019). Typically, prevalence of a necrotrophic effector gene in a particular *P. nodorum* population is thought to be driven by the prevalence of the corresponding wheat susceptibility gene target (Richards et al. 2019). However, higher prevalence of *SnTox1* in *P. nodorum* and lower prevalence of *Snn1* in global wheat germplasm led to the hypothesis that SnTox1 may have another

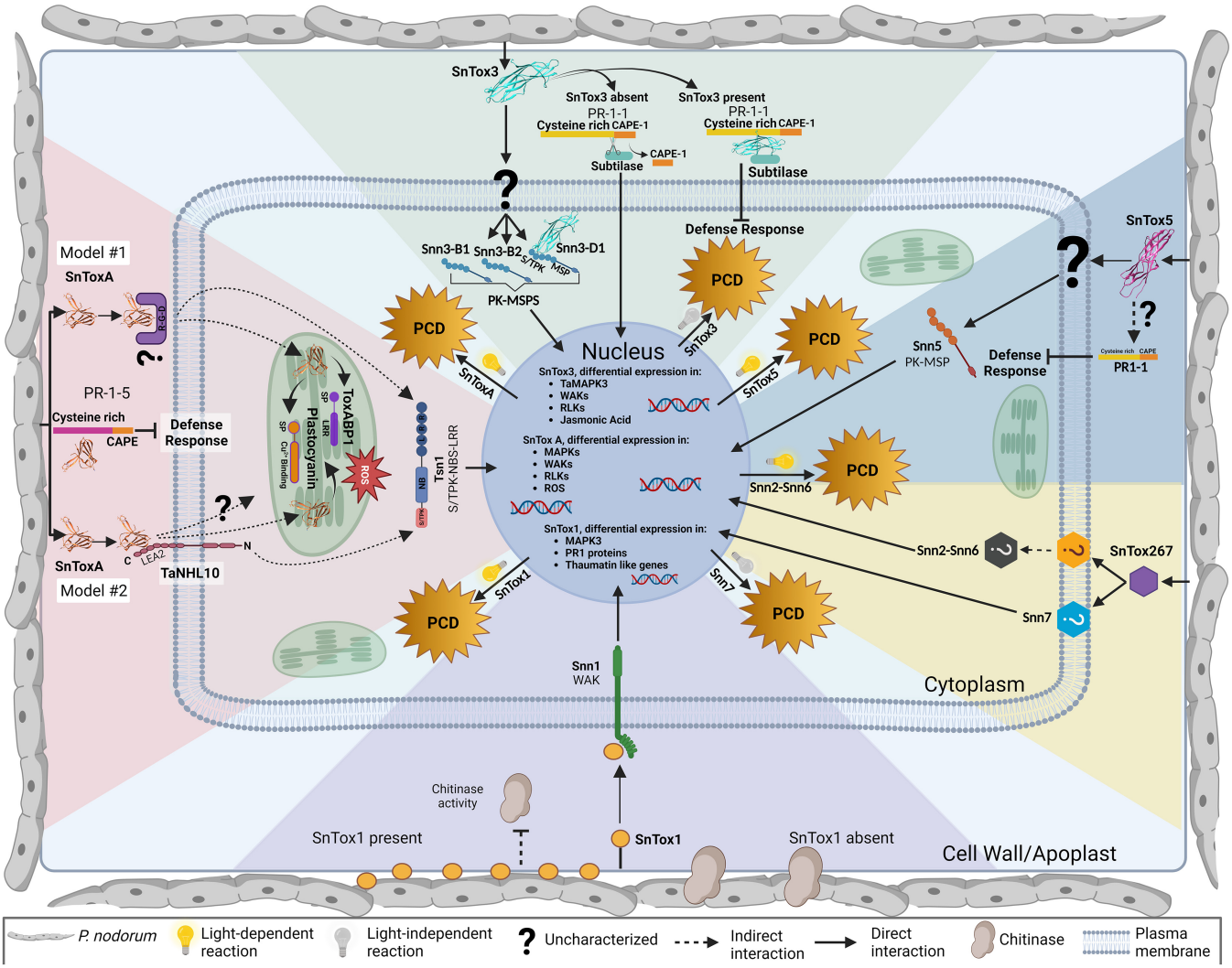

Fig. 2. Schematic review of known *Parastagonospora nodorum* necrotrophic effector proteins, their host interactors, and their predicted functions. SnTox1 (purple section) directly interacts with Snn1, which is a wall-associated kinase (WAK), and the interaction induces transcriptional changes in defense-related genes, including *TaMAPK3*, *PR1*, and thaumatin-like protein genes, to induce programmed cell death (PCD). Once the PCD and plant defenses are activated, SnTox1 binds chitin in the fungal cell wall, preventing cell wall damage by wheat chitinases. SnToxA (pink section) has two proposed models for its mode of action. Model 1: SnToxA is internalized into the cell through the interaction of the RGD domain of SnToxA with a yet-unidentified transmembrane integrin-like receptor. Internalized SnToxA is localized into the chloroplast and interacts directly with ToxAPB1 and plastocyanin. SnToxA indirectly interacts with Tsn1 and activates *MAPK*s, *WAK*s, and *RLK*s and also downregulates genes involved in detoxifying reactive oxygen species (ROS) in chloroplasts to cause PCD. Model 2:SnToxA directly interacts with the LEA2 domain of TaNHL10, which is a membrane-bound protein, and it indirectly interacts with Tsn1 to induce SnToxA-Tsn1-mediated PCD. In both proposed models, SnToxA interacts with PR-1-5, possibly to suppress host defenses. SnTox267 (yellow section) triggers PCD using multiple pathways; the SnTox267-Snn2/Snn6 pathway is strictly light dependent, and the SnTox267-Snn7 pathway is light independent. SnTox3 (green section) interacts with Snn3, a protein with protein kinase (PK) and major sperm motility (MSP) domains and reprograms the host transcription to induce light-independent PCD. In addition, SnTox3 also interacts directly with PR1-1 and inhibits the cleavage of the CAPE-1 peptide by subtilase, preventing the CAPE-1-mediated defense response. SnTox5 (blue section) interacts with Snn5, a PK-MSP protein, to induce PCD in a light-dependent manner, and because of the structural homology with SnTox3, it is also predicted to interact with PR-1 class proteins to modulate defense. Created with http://www.BioRender.com.

function in addition to the induction of PCD (Liu et al. 2012). SnTox1 was subsequently shown to possess sequence motifs that were associated with plant chitin-binding proteins. SnTox1 bound to both free chitin and chitin in the fungal cell wall and protected the pathogen from wheat chitinases with and without chitin-binding domains, showing a secondary, or possibly the primary, function of this effector (Liu et al. 2016; Fig. 2; Supplementary Fig. S1). Finally, the comparison of infection processes of the GFP-tagged avirulent strain and its *SnTox1* transformant showed that SnTox1 may play an important role in fungal penetration (Liu et al. 2012).

In summary, SnTox1 facilitates the penetration of *P. nodorum* into the leaf tissue by directly targeting *Snn1*, a PRR-like gene, to trigger host defense responses, including PCD, but to the advantage of the necrotrophic pathogen. Once PCD and other defense responses are triggered, SnTox1 is involved in binding to the chitin in its own cell wall to protect from wheat chitinases expressed during defense (Fig. 2; Supplementary Fig. S1).

SnToxA, a necrotrophic effector that is used by multiple wheat pathogens

ToxA was the first proteinaceous necrotrophic effector identified in any plant pathogen, and to date, the *ToxA* gene has been identified in three necrotrophic fungal pathogens of wheat including *Pyrenophora tritici-repentis* (*Ptr ToxA*), *P. nodorum* (*SnToxA*), and *Bipolaris sorokiniana* (*BsToxA*) (Friesen et al. 2006, 2018; McDonald et al. 2018; Tomás and Bockus 1987). ToxA was originally purified from culture filtrates of *P. tritici-repentis* and it was characterized as a small, secreted protein with a molecular weight of 13.2 kDa (Ballance et al. 1989; Tomás and Bockus 1987). ToxA consisted of a 23-aa signal peptide and a 37-aa pro-sequence that was cleaved before the secretion of the 178-aa mature protein (Sarma et al. 2005; Tuori et al. 2000). Ciuffetti et al. (1997) cloned the *ToxA* gene in *P. tritici-repentis* and validated the gene using gain-of-function transformants. *ToxA* was a single-copy, 534-bp gene with two introns. Subsequently, a homolog of *Ptr ToxA* with 99.7% homology was identified in the newly acquired *P. nodorum* genome sequence (Hane et al. 2007), and diversity studies showed that *ToxA* likely originated in *P. nodorum* before being horizontally transferred into *P. tritici-repentis* (Friesen et al. 2006; Ghaderi et al. 2020; McDonald et al. 2019).

ToxA targets the host susceptibility gene *Tsn1* to induce PCD. The *ToxA-Tsn1* interaction is light dependent and plays a major role in disease in both seedling and adult plant stages of SNB (Chu et al. 2010; Faris and Friesen 2009; Faris et al. 2011; Friesen et al. 2008; Kariyawasam et al. 2018; Liu et al. 2006). Of the SNB necrotrophic interactions, the *ToxA-Tsn1* interaction is the most extensively characterized. ToxA contains a conserved RGD (Arg-Gly-Asp) motif that was suggested to be involved in protein–protein interactions (Meinhardt et al. 2002; Sarma et al. 2005). Site-directed mutants of the RGD domain failed to induce necrosis, suggesting that the RGD domain was involved in PCD (Meinhardt et al. 2002). Competition assays showed that co-infiltration of the RGD tripeptide and Ptr ToxA reduced PCD, and it was hypothesized that the RGD motif potentially interacted with a transmembrane integrin-like receptor before internalizing into the cytoplasm (Manning et al. 2004; Meinhardt et al. 2002). ToxA interacted directly with plastocyanin (Tai et al. 2007) and ToxABP1 (Manning et al. 2007), both of which were chloroplast-localized proteins, supporting that ToxA likely localized to the chloroplast once it was internalized into the cytoplasm of the mesophyll cells (Manning and Ciuffetti 2005; Manning et al. 2008; Fig. 2; Supplementary Fig. S2).

Dagvadorj et al. (2022) showed that ToxA directly interacted with the C-terminal LEA2 extracellular domain of TaNHL10, a wheat transmembrane protein and that the ToxA-TaNHL10 interaction was vital for ToxA-induced PCD. Dagvadorj et al. (2022) also used a yeast two-hybrid (Y2H) approach to reproduce the ToxA interaction with plastocyanin but not the ToxA-ToxABP1 interaction. Additionally, Dagvadorj et al. (2022) showed that ToxABP1 was autoactive, calling into question the results of Manning et al. (2007). Dagvadorj et al. (2022) also showed that the interaction of ToxA with TaNHL10 was not affected by the loss of the RGD motif and that the TaNHL10-ToxA interaction was critical for *Tsn1*-mediated necrosis. Dagvadorj et al. (2022) did not exclude the possibility of internalization of ToxA into the cytoplasm through TaNHL10, suggesting more research was required to draw these conclusions. In another Y2H study, Lu et al. (2014) showed that ToxA directly interacted with the pathogenicity related-1 (PR-1) protein PR-1-5, and the RGD motif was not necessary for this interaction (Fig. 2; Supplementary Fig. S2).

None of these studies showed a direct interaction between ToxA and Tsn1. Faris et al. (2010) showed that *Tsn1* encodes a tri-domain protein consisting of a serine/threonine protein kinase domain, a nucleotide-binding site (NBS), and a leucine-rich repeat (LRR) domain showing structural resemblance to NBS-LRR plant resistance genes. In the presence of *Tsn1*, Pandelova et al. (2009) showed that ToxA induced the downregulation of genes encoding for enzymes involved in detoxifying reactive oxygen species (ROS) associated with the chloroplast. Therefore, ROS accumulated in the chloroplasts, disrupting the protein homeostasis in both photosystems, eventually resulting in PCD (Manning et al. 2009; Pandelova et al. 2009). In addition, infiltration of ToxA induced the upregulation of defense-related genes such as *PR1-5*, *MAPKs*, wall-associated kinases (*WAK*s), and receptor-like kinases (*RLK*s) that are also known to play a role in triggering PCD (Adhikari et al. 2009; Lu et al. 2014; Fig. 2; Supplementary Fig. S2). The fact that *Tsn1* is a resistance-like gene and that transcriptional changes appeared to be defense related provides evidence that ToxA is likely manipulating an ETI pathway to induce PCD to the advantage of the pathogen (Faris and Friesen 2020; Friesen and Faris 2021).

SnTox267 has multiple wheat susceptibility targets

SnTox2-*Snn2* (Friesen et al. 2007), SnTox6-*Snn6* (Gao et al. 2015), and SnTox7-*Snn7* (Shi et al. 2015) were three proposed necrotrophic effector–susceptibility gene interactions that were identified in three independent quantitative trait loci (QTL) mapping studies. *Snn2*, *Snn6*, and *Snn7* were mapped to wheat chromosome arms 2DL, 6AL, and 2DS, respectively, suggesting three different host target genes. In addition, each of these studies showed that SnTox2, SnTox6, and SnTox7 were small, secreted proteins (Friesen et al. 2007; Gao et al. 2015; Shi et al. 2015).

Recently, a genome-wide association study (GWAS) involving 197 *P. nodorum* isolates showed that a single virulence locus was associated with the *Snn2* and *Snn6* wheat differential lines (Richards et al. 2022). The most significantly associated marker was located adjacent to a gene encoding for a candidate effector. Disruption of the candidate gene eliminated the disease association with both susceptibility genes, revealing that SnTox2 and SnTox6 were the same protein interacting with *Snn2* and *Snn6* (Richards et al. 2022). In the same study, an F_2 population that segregated for *Snn7* was infiltrated with the same necrotrophic effector and sensitivity mapped to the *Snn7* locus, suggesting that Snn7 was also targeted by the same necrotrophic effector as Snn2/Snn6, which was therefore named SnTox267 (Richards et al. 2022). Evaluation of multiple genetic crosses showed that both *Snn2* and *Snn6* were required for the induction of PCD by SnTox267, suggesting that *Snn2* and *Snn6* were components of the same pathway targeted by SnTox267 (Fig. 2; Supplementary Fig. S3). In addition, previous studies showed that the PCD resulting from the SnTox267-*Snn2*/*Snn6* interaction was

strictly light dependent; however, the PCD resulting from the SnTox267-*Snn7* interaction was not light dependent, suggesting that SnTox267 was hijacking at least two different pathways to induce PCD (Fig. 2; Supplementary Fig. S3).

SnTox267 is a 265-aa protein with 10 cysteine residues encoded by a 798-bp single exon gene located on *P. nodorum* chromosome 14 (Richards et al. 2022). SnTox267 has a 16-aa signal peptide that gets cleaved to form a 27.4-kilodalton (kDa) mature protein. Interestingly, like *SnTox1*, *SnTox267* was detected in ~95% of the North American isolates used in the Richards et al. (2022) GWAS study. This high prevalence of SnTox267 suggested a high level of sensitivity in popular wheat cultivars or, like SnTox1 and SnTox3 (see SnTox1 and SnTox3 sections above and below, respectively), a secondary function important to the pathogen's fitness. In-depth studies are required to further characterize the potential of additional beneficial modes of action of SnTox267.

SnTox3, a dual-function protein with multiple host targets

SnTox3 is another small, secreted necrotrophic effector that *P. nodorum* uses to target wheat genotypes with the corresponding wheat susceptibility gene *Snn3*. The SnTox3-*Snn3* interaction was first characterized by Friesen et al. (2008), and the original *Snn3* gene was mapped to the short arm of wheat chromosome 5B. The wheat gene was later renamed *Snn3-B1* following the discovery of a homoeologous susceptibility gene, *Snn3-D1*, on the short arm of chromosome 5D (Zhang et al. 2011). The SnTox3-*Snn3-B1* interaction was a light-independent interaction that played an important role in disease at the seedling and adult plant stages in the greenhouse and field, respectively (Friesen et al. 2008; Ruud et al. 2017).

SnTox3 was a 693-bp intron-free gene that encoded a 230-aa protein with a predicted mass of 25.85 kDa (Liu et al. 2009). SnTox3 consisted of a 20-aa signal peptide and a 52-aa prosequence that was removed by the Kex2 protease before the secretion of the mature SnTox3. The elucidation of the crystal structure of the SnTox3 protein showed eight β-strands arranged in an anti-parallel formation to form a β-barrel structure. Three disulfide bonds that formed between cysteine residues were also detected between these β strands. In addition to the eight β strands, SnTox3 also harbored two additional β strands that formed a β-hairpin-like structure (Outram et al. 2021; Fig. 2; Supplementary Fig. S4).

Liu et al. (2009) showed that in planta expression of *SnTox3* peaked at 72 hpi, and Winterberg et al. (2014) showed that SnTox3 reprogrammed host transcription to upregulate genes involved in host defense, including mitogen-activated protein kinase (MAPK) pathway genes, pathogenesis related (PR) genes, jasmonic acid pathway genes, and phenylpropanoid pathway genes, to induce PCD. In fact, like SnTox1, SnTox3 also induced the upregulation of *TaMAPK3*, suggesting that SnTox3 also manipulates PTI pathways to induce PCD (Winterberg et al. 2014). *Snn3-D1* encoded for a protein with protein kinase (PK) and major sperm protein (MSP) domains, and mutant analysis showed that both domains played a critical role in PCD induction (Zhang et al. 2021). Not much is known about the functional role of the MSP in perception of SnTox3 (Fig. 2; Supplementary Fig. S4).

Breen et al. (2016) showed that SnTox3 interacted directly with the wheat pathogenicity related-1 (PR1) protein TaPR1-1. Furthermore, this experiment revealed that the interaction happened at the C-terminal region of the TaPR1-1 that consisted of a defense-related signaling peptide designated CAPE1. Sung et al. (2021) subsequently showed that the mutation of SnTox3^{P173S} weakened or abolished the interaction with TaPR1. The residue P173 is a surface-exposed residue localized to β-strand 7. SnTox3^{P173S} was able to induce necrosis on susceptible wheat genotypes upon infiltration, suggesting that the SnTox3-TaPR1 interaction is independent of the SnTox3-Snn3-induced PCD. In another mutant analysis study, Outram et al. (2021) showed that SnTox3 mutations at R129, D152, R196, E217, S218, T160, P162, and R205 prevented SnTox3-Snn3-induced PCD, suggesting that one or more of these regions may interact with Snn3. However, Y2H analysis showed that these mutants still interacted with TaPR1-1, suggesting that different regions/surfaces of the protein were mediating the SnTox3-Snn3 and the SnTox3-TaPR1 interactions. Infiltration of TaPR1 before the inoculation of *P. nodorum* led to a reduction in symptoms, suggesting that TaPR1 plays a role in resistance signaling against *P. nodorum* (Sung et al. 2021). In addition, unlike the wild-type PR1, inoculation of *P. nodorum* on a leaf region infiltrated with a TaPR1 lacking the CAPE1 domain showed no reduction in disease symptoms, providing further evidence that the CAPE1 peptide plays a role in TaPR1-mediated resistance against *P. nodorum.*

When TaPR1-1 was pre-infiltrated alone on wheat lines lacking *Snn3*, followed by inoculation of *P. nodorum*, TaPR1-1 induced a reduction in disease symptoms; however, the co-infiltration of TaPR1-1 and SnTox3 under the same conditions showed that TaPR1-1 failed to reduce disease symptoms, showing a role of SnTox3 even in the absence of *Snn3*. When both TaPR1-1 and SnTox3 ^{P173S} (mutant that does not interact with TaPR1-1) were pre-infiltrated, the ability of TaPR1-1 to reduce disease symptoms was restored, showing that direct interaction between SnTox3 and TaPR1 is required to suppress the TaPR1-mediated defense response (Sung et al. 2021). Finally, Sung et al. (2021) showed that apoplastic proteases cleaved the CAPE1 peptide. However, the binding of SnTox3 to the PR1-1 protein inhibits this cleavage and disrupts the defense response signaling, which collectively suggests a role of defense suppression followed by defense activation (PCD) for SnTox3 (Fig. 2; Supplementary Fig. S4).

SnTox5 induces PCD but also facilitates colonization of the mesophyll layer

SnTox5 is a necrotrophic effector that targets wheat genotypes with the susceptibility gene *Snn5* on chromosome arm 4BL in a light-dependent manner (Friesen et al. 2012). A GWAS analysis showed that a single virulence locus was associated with *Snn5*-mediated susceptibility, with the most significant marker–trait association being identified in the 654-bp single exon gene *Sn2000_06735* (Kariyawasam et al. 2022). This gene, which was predicted to be an effector, was validated using CRISPR-Cas9-mediated gene disruption and gain-of-function transformants and eventually designated as *SnTox5*. It encoded a 217-aa small, secreted, cysteine-rich (six cysteines) protein that consisted of a putative 22-aa signal peptide and, like SnTox3, a 49-aa pro-sequence that is predicted to be cleaved by the Kex2 protease before the secretion of the mature 16.26-kDa protein. The predicted 3D structure of SnTox5 consisted of 11 β strands, with 8 of them predicted to form a β-barrel, like SnTox3 (Kariyawasam et al. 2022). In fact, despite the amino acid sequence homology of only 45.13%, 98% structural homology was observed between the mature proteins of SnTox3 and SnTox5 (Fig. 2; Supplementary Figs. S4 and S5).

SnTox5 expression peaked at 24 hpi during colonization of the epidermis but before colonization of the mesophyll, which began at 48 hpi (Kariyawasam et al. 2022). Confocal microscopy showed that hallmarks of PCD such as chloroplast deterioration and chlorophyll degradation were not observed until 72 hpi, indicating that PCD was delayed until the pathogen was able to build biomass by colonizing the mesophyll cell layer (Kariyawasam et al. 2022). Interestingly, wild-type isolates expressing SnTox5 were able to colonize wheat lines, even in the absence of *Snn5*; however, *SnTox5*-disruption mutants failed to colonize the mes-

ophyll (Kariyawasam et al. 2022). Therefore, early expression of *SnTox5*, the ability of SnTox5-expressing isolates to colonize mesophyll of non-*Snn5* lines, and the inability of SnTox5 mutants to colonize the mesophyll indicate that SnTox5 is important for mesophyll colonization that is independent of PCD induction.

Role of *P. nodorum* effectors in the infection process

Traditional effectors are typically involved in delaying or modulating the host defense response or keeping the pathogen unrecognized during infection, thus improving its efficiency in completing its pathogenic life cycle. *P. nodorum* is a necrotrophic pathogen that appears to thrive in a defense response-induced necrotic environment (Adhikari et al. 2009; Pandelova et al. 2009; Winterberg et al. 2014). However, *P. nodorum* is not a brute-force pathogen that just kills cells and feeds on the nutrients; rather, it is more elegant in its infection process, in that it effectively modulates the spatial and temporal aspects of the host defense to its advantage.

Initially, *P. nodorum* deploys multiple cell wall-degrading enzymes (CWDEs) that allow initial penetration of the leaf (Carlile et al. 2000; Lalaoui et al. 2000; Lehtinen 1993; Magro 1984). These CWDEs act as effectors and include cutinases, cellulases, pectin lyases, xylanases, and glucanases; however, these enzymes are not known to independently induce necrosis (Friesen and Faris 2010). *P. nodorum* can penetrate through stomata (Solomon et al. 2006a) or directly between epidermal cells (Liu et al. 2012) to gain access to the epidermal layer, where it remains intercellular, surrounding the epidermal cells before moving to the mesophyll layer, where it also remains intercellular (Kariyawasam et al. 2022).

Once the colonization of the epidermal layer is complete, *P. nodorum* continues its colonization of the mesophyll layer by radiating out from the initial penetration of the epidermis; however, very little cell death is visible until colonization is complete, indicating the use of effectors to delay defense activation while building biomass. Following colonization, PCD is triggered (Kariyawasam et al. 2022). The PCD induced by SnToxA (Friesen et al. 2006), SnTox1 (Liu et al. 2012), SnTox267 (Friesen et al. 2007), and SnTox5 (Friesen et al. 2012) is light dependent, suggesting the involvement of the chloroplast. Chloroplasts are known to be major ROS producers, driving hypersensitive response-mediated PCD (Mur et al. 2008). Downregulation of detoxifying ROS in chloroplast upon ToxA-Tsn1 interaction (Pandelova et al. 2009), the observation of the oxidative burst associated with the SnTox1-*Snn1* interaction (Liu et al. 2012), and the observation of deteriorating chloroplast in confocal microscopy (Kariyawasam et al. 2022) lead us to speculate that ROS originates in the chloroplast, and the accumulation of ROS results in chloroplast deterioration. This oxidative burst ultimately leads to the breakdown of organellar and cellular membranes, making cellular nutrients available to the pathogen, which has already colonized the apoplast surrounding these cells.

Necrotrophic effectors target host genes to induce PCD (as described earlier) but have repeatedly been shown to have additional functions that contribute to their in planta fitness. In addition to targeting *Snn1* to induce PCD, SnTox1 binds to chitin in the fungal cell wall, protecting *P. nodorum* from wheat chitinases upregulated during the defense response (Liu et al. 2012, 2016), indicating that SnTox1 is involved in protecting the pathogen cell wall from enzymatic degradation during its penetration and colonization phases. SnTox3 binds to TaPR-1 proteins to prevent the cleavage of a CAPE1 peptide that is involved in defense signaling (Breen et al. 2016; Sung et al. 2021) even in the absence of *Snn3*. SnTox5 facilitates the colonization of the mesophyll layer, even in the absence of the susceptibility target *Snn5*, and although not experimentally shown, SnToxA may also be involved in some aspect of defense modulation based on its ability to bind a PR-1-5 protein (Dagvadorj et al. 2022; Lu et al. 2014). Collectively, evidence suggests a role of several known necrotrophic effectors not only in triggering PCD, but also in modulating negative aspects of the host defense response (Fig. 2).

Available Resources and Tools

Many genetic and genomic resources have been developed to study the *P. nodorum*–wheat pathosystem. Genetic marker data for host resistance can be accessed through the T3/Wheat (https://wheat.triticeaetoolbox.org) (Morales et al. 2022) and GrainGenes (https://wheat.pw.usda.gov/GG3) platforms.

Genomic resources, including reference-quality genome assemblies of *P. nodorum* (Bertazzoni et al. 2021; Hane et al. 2007; Richards et al. 2018; Syme et al. 2013, 2016), resequencing of more than 200 globally collected *P. nodorum* strains (Richards et al. 2019; Syme et al. 2018), and RNA sequencing (Jones et al. 2019) and chromatin immunoprecipitation sequencing data of *P. nodorum* (John et al. 2022) can be found under BioProjects at the National Center for Biotechnology and Information (NCBI, www.ncbi.nlm.nih.gov) and other repositories such as Zenodo and GitHub (Table 1). The availability of genomic data has made it possible for bioinformatic tools such as GAPIT v. 3.0 (Huang et al. 2019), SignalP v. 6.0 (Teufel et al. 2022), and EffectorP v. 3.0 (Sperschneider and Dodds 2022) to be used extensively to mine putative effectors (Richards et al. 2018; Wyatt et al. 2020).

Many molecular tools such as polyethylene glycol-mediated transformation (Cooley et al. 1988; Liu and Friesen 2012), split-marker-mediated gene disruption (Liu et al. 2009, 2012; Richards et al. 2022; Solomon et al. 2006b); CRISPR-Cas9-mediated gene disruption and gene editing (Kariyawasam et al. 2022; Khan et al. 2020), quantitative PCR (Kariyawasam et al. 2022; Liu et al. 2009, 2012; Richards et al. 2022; Solomon et al. 2004), confocal microscopy (Kariyawasam et al. 2022; Liu et al. 2016; Nelson et al. 2023; Solomon et al. 2006c), Y2H (Dagvadorj et al. 2022; Lu et al. 2014; Shi et al. 2016; Sung et al. 2021), co-immunoprecipitation (Sung et al. 2021), and mutant analysis (Lu et al. 2014; Outram et al. 2021) have been used to characterize the *P. nodorum*–wheat interaction. These tools can be used in the future to address research gaps, as they have already been optimized.

Knowledge Gaps and Future Research

Of the five effector genes cloned, *SnToxA*, *SnTox1*, and *SnTox3* have been the most extensively studied at the molecular level. Structures have been determined experimentally for SnToxA (Sarma et al. 2005) and SnTox3 (Outram et al. 2021), and protein interactors from the host have been identified and characterized to various levels for SnToxA (Lu et al. 2014, Dagvadorj et al. 2022; Manning et al. 2007; Tai et al. 2007), SnTox1 (Shi et al. 2016), and SnTox3 (Outram et al. 2021, Sung et al. 2021). In addition, cytoplasmic and apoplastic localization of SnToxA (Manning and Ciuffetti 2005) and SnTox1 (Liu et al. 2016), respectively, were reported. However, protein–protein interactions and the localization of SnTox267 and SnTox5 have yet to be investigated. Therefore, Y2H, co-immunoprecipitation, and confocal microscopy studies using fluorescently tagged fusion proteins need to be performed for SnTox267 and SnTox5 to provide a more complete understanding of these necrotrophic effectors and how they target the host.

Faris et al. (2010) showed that SnToxA does not interact directly with its susceptibility target *Tsn1*. This presented the question of the role of *Tsn1* in PCD induced by SnToxA, a question

yet to be answered. Discovery of TaNHL10 added to our knowledge of this interaction but at the same time complicated the interaction of ToxA, Tsn1, and the ToxA and Tsn1 intermediate interactors. In fact, a direct interaction between a necrotrophic effector and its corresponding susceptibility gene has been demonstrated only for the SnTox1-Snn1 interaction (Shi et al. 2016). Identification of two host genes in the same pathway that are essential for the induction of PCD by SnTox267 (Richards et al. 2022) provides the first evidence that these "susceptibility" genes can act in different parts of a single pathway rather than acting as direct receptors like that of *Snn1*. Therefore, it is important to identify the rest of the susceptibility genes and characterize their interaction with the corresponding effectors, both genetically and biochemically, in order to elucidate the role of the effector and the known susceptibility gene.

To date, transcriptional changes induced in wheat upon the establishment of SnToxA-Tsn1 (Pandelova et al. 2009) and SnTox3-Snn3 (Winterberg et al. 2014) interactions have been characterized. In each case, components of the host defense mechanism were up- and downregulated during the infection process. Analysis of transcriptional changes allows us to identify the host pathways manipulated by these effectors to induce PCD. Currently we are in the process of acquiring and analyzing the transcriptional changes in susceptible wheat induced by SnTox267 and SnTox5.

Characterized necrotrophic effector–susceptibility gene interactions represent only a subset of marker–trait associations observed in QTL mapping or GWAS analysis (Peters Haugrud et al. 2022). This suggests the possibility of other unidentified necrotrophic effector–susceptibility gene interactions or more general resistance mechanisms not necessarily directly related to necrotrophic effectors. Therefore, it is important to continue to evaluate the genetics of resistance/susceptibility in diverse wheat germplasm. Necrotrophic effectors are only a fraction of the effectorome of *P. nodorum*. Few effectors that are not involved in PCD have been functionally characterized in the *P. nodorum*–wheat pathosystem. The available molecular and bioinformatics tools can be used to characterize these putative effectors to get a complete picture of the infection process.

To conclude, *P. nodorum* has indeed been shown to be a master manipulator of the host defense by using both necrotrophic effectors that induce PCD and traditional effectors that modulate the defense response to the advantage of the pathogen. However, there are several questions yet to be answered to gain a complete understanding of this pathosystem. Comprehensive understanding of this pathosystem will eventually allow us to deploy economically sound management strategies against *P. nodorum*.

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Table 1. Genomic resources available for *Parastagonospora nodorum*

Database	BioProject	Genomic resource	Citation	Link
NCBI	PRJNA13754, PRJNA21049,	Reference quality genome of Australian isolate SN15	Hane et al. 2007	https://www.ncbi.nlm.nih.gov/bioproject/13754 https://www.ncbi.nlm.nih.gov/bioproject/21049
	PRJNA170816	Resequencing of Sn79-1087	Syme et al. 2013	https://www.ncbi.nlm.nih.gov/bioproject/170816
	PRJNA476481	Resequencing of 10 *Parastagonospora avenae*, 18 *P. nodorum*, and 2 *Phaeospharia* sp. isolates	Syme et al. 2018	https://www.ncbi.nlm.nih.gov/bioproject/476481
	PRJNA398070	Reference quality genomes of *P. nodorum* strains LDN03-Sn4, Sn2000, Sn79-1087	Richards et al. 2018	https://www.ncbi.nlm.nih.gov/bioproject/398070
		Resequencing of 198 *P. nodorum* isolates	Richards et al. 2019	
	PRJNA612761	Resequencing of 153 *P. nodorum* Western Australian isolates	Jones et al. 2021	https://www.ncbi.nlm.nih.gov/bioproject/612761
	PRJNA632579	Transcriptome of SN15 and *PnPf2* mutant of SN15	Jones et al. 2019	https://www.ncbi.nlm.nih.gov/bioproject/632579
	PRJNA686477	Reference-quality genome of Australian isolates SN15	Bertazzoni et al. 2021	https://www.ncbi.nlm.nih.gov/bioproject/686477
	PRJNA824526	Chromatin immunoprecipitation sequencing data for PnPf2 transcription factor	John et al. 2022	https://www.ncbi.nlm.nih.gov/bioproject/824526
Zenodo	–	*P. nodorum* de novo assemblies of 197 *P. nodorum* isolates	Richards et al. 2022	https://zenodo.org/records/4560540
	–	De novo gene annotation of *P. nodorum* reference isolate Sn2000	Kariyawasam et al. 2023b	https://zenodo.org/record/7768313#.ZB30JLLMJyG
	–	De novo gene annotation of *P. nodorum* reference isolate Sn79-1087	Kariyawasam et al. 2023a	https://zenodo.org/record/7768408#.ZB30lLLMJyE
GitHub	–	LDN03-Sn4 genome annotation LDN03-Sn4 reference-quality genome sequence	Richards et al. 2022	https://github.com/jkzrich/pnodorum_popgen

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