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Proteinaceous necrotrophic effectors in fungal virulence

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Abstract. The host–pathogen interface can be considered as a biological battlefront. Molecules produced by both the pathogen and the host are critical factors determining the outcome of the interaction. Recent studies have revealed that an increasing number of necrotrophic fungal pathogens produce small proteinaceous effectors that are able to function as virulence factors. These molecules can cause tissue death in host plants that possess dominant sensitivity genes, leading to subsequent pathogen colonisation. Such effectors are only found in necrotrophic fungi, yet their roles in virulence are poorly understood. However, several recent key studies of necrotrophic effectors from two wheat (*Triticum aestivum* L.) pathogens, *Pyrenophora tritici-repentis* (Died.) Drechs. and *Stagonospora nodorum* (Berk.) Castell. & Germano, have shed light upon how these effector proteins serve to disable the host from the inside out.

Additional keywords: host-selective toxin, net blotch, Pyrenophora, septoria, Stagonospora, tan spot.

Introduction

Necrotrophic fungi were traditionally considered as non-host-specific pathogens that use a large array of cell wall-degrading enzymes and non-specific toxins for pathogenicity (Hammond-Kosack and Rudd 2008). It is now known that some of these necrotrophs possess an arsenal of effectors used to disable susceptible hosts ahead of colonisation. Necrotrophic effectors share some common properties with the avirulence (Avr) effectors of biotrophic fungal pathogens (reviewed elsewhere in this issue) (Table 1). However, in contrast to the classical gene-for-gene hypothesis, where the interaction of avirulence effectors with host resistance (R)-gene complexes leads to resistance, necrotrophic effectors function in an 'inverse' manner. An interaction between a necrotrophic effector and the product of a host dominant sensitivity gene leads instead to disease (Fig. 1).

Necrotrophic effectors are a diverse group of molecules that induce tissue death in host plants possessing the appropriate genotype. They are typically small and can be proteinaceous in nature or secondary metabolites. Examples of metabolite-based effectors include victorin from *Cochliobolus victoriae* Nelson and AAL-toxin from *Alternaria alternate* (Fr. : Fr.) Keissl., and these have been extensively reviewed elsewhere (Walton 1996; Wolpert *et al*. 2002). The purpose of this review will focus on discussing recently discovered proteinaceous necrotrophic effectors and their roles in virulence.

Effectors of *Pyrenophora tritici* f. sp. *repentis*

Pyrenophora tritici-repentis (Died.) Drechs. is the causal agent of tan spot (previously called yellow spot or yellow leaf spot), a devastating disease of wheat (*Triticum aestivum* L.). To date, two proteinaceous effectors of this necrotroph have been identified, PtrToxA and PtrToxB, which are encoded by the genes *PtrToxA* and *PtrToxB*.

PtrToxA was the first effector to be isolated and is the best characterised. The PtrToxA protein is the product of a single copy gene that is present in ~80% of a worldwide collection of isolates (Friesen *et al*. 2006). PtrToxA is a small (13.2 kDa) secreted protein that causes necrosis in sensitive wheat genotypes (Ballance *et al*. 1989; Tomas *et al*. 1990; Tuori *et al*. 1995). Ciuffetti *et al*. (1997) demonstrated that the *PtrToxA* gene is both necessary and sufficient for the pathogenicity of *P. tritici-repentis*, since transformation of a non-pathogenic *P. tritici-repentis* isolate with the *PtrToxA* gene was sufficient to render that isolate pathogenic on PtrToxA-sensitive wheat lines.

Wheat sensitivity to the PtrToxA effector is conditioned by *Tsn1*, a single gene present on the long arm of chromosome 5B (Faris *et al*. 1996; Anderson *et al*. 1999). The *Tsn1* gene has very recently been cloned and found to possess nucleotide-binding site (NBS), leucine-rich repeat (LRR) and serine/threonine protein kinase domains, all of which are necessary for PtrToxA sensitivity (Faris *et al*. 2010). Paradoxically, these domains are common features of plant disease R genes involved in defence against biotrophic pathogens (Martin *et al*. 2003).

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Table 1. Properties of proteinaceous avirulence and necrotrophic effectors

Characteristic	Avirulence effector	Necrotrophic effector
Relative small size[A]	Yes	Yes
Secreted	Yes	Yes
Location of recognition	Intra- or extracellular	Unknown
Cysteine-rich[B]	Predominantly	Some
Compatible host response	No disease	Disease
Fungal lifestyle	Biotrophic or hemibiotrophic	Necrotrophic
Function during host recognition	Hypersensitive response leading to pathogen containment. Mediated through direct (cognate resistance proteins) or indirect Avr recognition (guard proteins).	Host cell death leading to tissue necrosis. Recognition of effector by host dominant sensitivity proteins.
Role in virulence	Largely unknown but some Avr proteins function as protease inhibitors and binds chitin – protection against plant chitinases.	Largely unknown but some effectors manipulate the hosts' photosystem and plasma H^+ ATPase functions.

[A]Under 30 kDa.
[B]Greater than four cysteines per mature polypeptide.

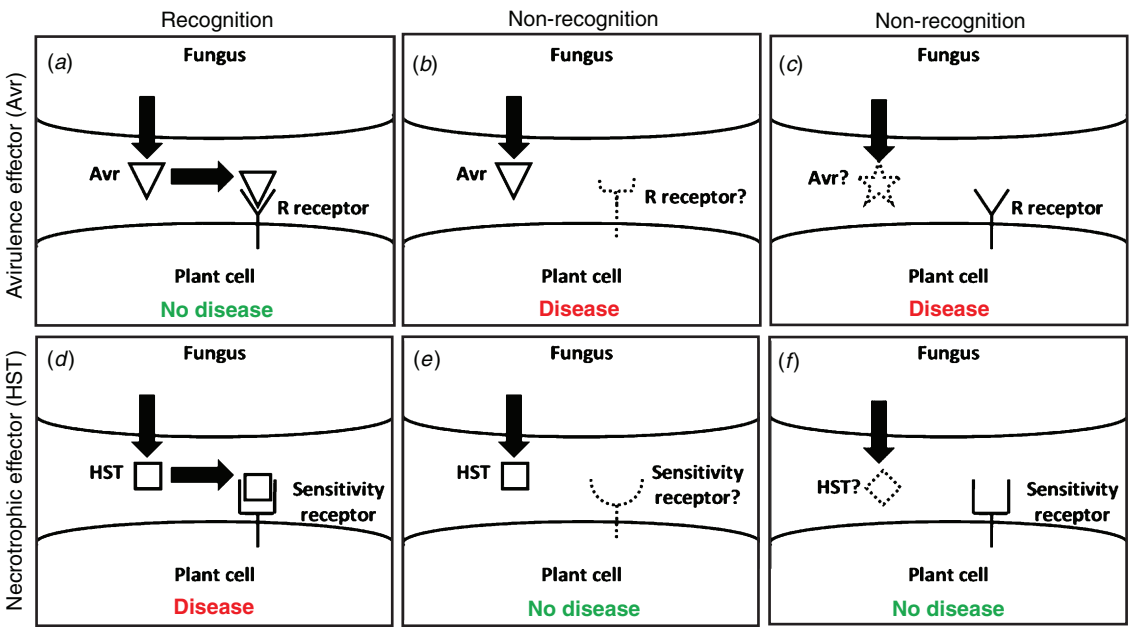

Fig. 1. Outcomes of fungal effector–host interactions. (*a*) to (*c*) depict the host response to a fungal Avr protein. The host will mount a successful defence response against the pathogen if Avr recognition occurs (*a*). Infection will occur if the pathogen is not recognised due to an absent or altered R (resistance) receptor (*b*) or Avr effector (*c*). Necrotrophic effectors function in an inverse manner (*d–f*). A successful infection will only occur during effector recognition (*d*). During a non-recognition event, no disease will result due to an absent or altered host sensitivity receptor (*e*) or fungal effector (*f*). For simplicity, receptors are illustrated on the cell wall. We acknowledge that some host receptors are located intracellularly.

Parallels can be drawn with the victorin effector of *C. victoriae*, the causal agent of Victoria blight disease in oats (*Avena sativa* L.) (Meehan and Murphy 1946). The *C. victoriae* susceptibility gene has been identified in *Arabidopsis thaliana* (L.) Heynh. as *LOV1*, which, like *Tsn1*, has a NBS-LRR structure and mediates responses associated with biotrophic disease resistance (Lorang *et al.* 2007). However, victorin rapidly induces resistance-like physiology in oats, including a respiratory burst and apoptotic-like cell death (Wolpert *et al.* 2002). Yet how can the elicitation of a 'resistance' response lead to disease susceptibility? What seems at first to be a contradiction in terms could in fact be accounted for by the lifestyle of the pathogen. Thus, an environment that would be unfavourable to biotrophic pathogens (such as programmed cell death generated by the plant host) would actually be favourable to pathogens with a necrotrophic lifestyle. This suggests that R genes could paradoxically play a role in disease susceptibility by serving as targets for necrotrophic effectors such as PtrToxA.

Several recent studies have helped to unravel the mode of action of PtrToxA. Cytological analyses have revealed that PtrToxA is rapidly internalised into the mesophyll cells of sensitive wheat cultivars (Manning and Ciuffetti 2005;

Manning *et al*. 2008). However, the protein or proteins with which PtrToxA interacts at the cell membrane remain unidentified. Although *Tsn1* is necessary to mediate PtrToxA recognition, yeast two-hybrid experiments suggest that Tsn1 does not interact directly with PtrToxA, nor does it possess any apparent transmembrane domains (Faris *et al*. 2010).

Analysis of the mature PtrToxA protein sequence has demonstrated the presence of an arginyl-glycyl-aspartic (RGD) motif present at the surface of the PtrToxA protein (Zhang *et al*. 1997), which is required for PtrToxA internalisation (Manning and Ciuffetti 2005; Manning *et al*. 2008). This sequence is located on a solvent-exposed loop and is easily accessible for protein–protein interactions (Sarma *et al*. 2005*b*). In animals, the RGD motif is involved in the binding of extracellular matrix proteins to transmembrane integrin proteins (Ruoslahti and Pierschbacher 1986; D'Souza *et al*. 1991). These integrins have been utilised by many mammalian pathogens as adhesion sites and as binding sites for effectors (Isberg and Tran Van Nhieu 1994). Thus, it is it conceivable that PtrToxA internalisation relies on recognition of the RGD motif by a plant integrin-like protein receptor. Indeed, integrin-like proteins have been identified in plants (Faik *et al*. 1998; Labouré *et al*. 1999; Nagpal and Quatrano 1999; Swatzell *et al*. 1999; Sun *et al*. 2000; Baluska *et al*. 2003) and may provide candidates for mediating PtrToxA internalisation.

Much of what occurs once PtrToxA is internalised is still unknown. However, there is evidence to suggest that the action of PtrToxA is associated with photosynthetic pathways (Manning *et al*. 2009). For example, once inside the cell, the chloroplast appears to be a target for PtrToxA. *In vitro* experiments suggest that PtrToxA is able to interact with the chloroplast-localised protein called ToxA-binding protein 1 (ToxABP1), homologues of which have been found across several plant species (Sarma *et al*. 2005*a*; Manning *et al*. 2007). Although the precise function of ToxABP1 is unknown, it has been suggested that it plays a part in photosystem function or thylakoid formation (Wang *et al*. 2004; Keren *et al*. 2005). Indeed, PtrToxA treatment has been demonstrated to induce changes in PSI and PSII, leading to light-dependent accumulation of reactive oxygen species (ROS) in the chloroplast (Manning *et al*. 2009). The link between PtrToxA and photosynthesis is further supported by the light-dependent nature of PtrToxA-induced necrosis and the tight regulation of *Tsn1* transcription by both the circadian clock and light (Manning and Ciuffetti 2005; Faris *et al*. 2010).

Two independent studies have examined the global transcriptional changes induced by PtrToxA on sensitive wheat cultivars (Adhikari *et al*. 2009; Pandelova *et al*. 2009). Both studies illustrate that considerable transcriptional reprogramming occurs following PtrToxA treatment. Numerous defence-related host genes were upregulated at both early and late time points, including those associated with the phenylpropanoid pathway, lignification and ROS production, as well as genes functioning in signal transduction. Taken together, these studies suggest that PtrToxA disrupts photosynthetic electron transport, leading to ROS accumulation and plant cell death upon light exposure, thus creating an environment in which necrotrophic pathogens may thrive.

Another effector from *P. tritici-repentis* that has been characterised is PtrToxB. Like PtrToxA, PtrToxB is also a small secreted protein (6.6 kDa) which causes chlorosis on sensitive wheat genotypes and is encoded by a multicopy gene, *PtrToxB* (Orolaza *et al*. 1995; Strelkov *et al*. 1999; Martinez *et al*. 2001). Although not as prevalent as PtrToxA, PtrToxB has been found to be produced by several isolates around the world (Ali and Francl 2003; Friesen *et al*. 2005; Lamari *et al*. 2005). Wheat sensitivity is conditioned by the dominant *Tsc2* gene, which has been mapped to the short arm of chromosome 2B (Strelkov *et al*. 1999; Friesen and Faris 2004). Unlike most effectors, PtrToxB homologues have been found across a broad range of plant pathogenic ascomycetes, suggesting that it may have arisen in an early ancestor of the Ascomycota (Andrie *et al*. 2008). However, whether PtrToxB and its homologues play a role in plant–microbe interactions is yet to be elucidated.

Effectors of *Stagonospora nodorum*

Stagonospora nodorum (Berk.) Castell & Germano is the causal agent of stagonospora (previously septoria) nodorum blotch (SNB) in wheat (Solomon *et al*. 2006). Evidence of necrotrophic effectors produced by *S. nodorum* was first reported by Keller *et al.* (1994) using wheat embryos. Genes encoding effector proteins have only been identified and characterised recently (Friesen *et al*. 2006; Liu *et al*. 2009).

SnToxA was the first reported necrotrophic effector gene identified in *S. nodorum* (Friesen *et al*. 2006). A BLAST search of the *S. nodorum* genome sequence with *PtrToxA* identified an almost identical gene. Further genome exploration has revealed that *SnToxA* is located within a highly conserved genomic region of 11 kb that is present in both organisms. This 'transfercon' was hypothesised to be acquired by *P. tritici-repentis* from *S. nodorum* through lateral gene transfer, a biological process previously thought to be uniquely prokaryotic. This hypothesis is supported by several key pieces of evidence. Firstly, SNB has been known since the 1800s whilst tan spot was described as recently as 1941. Prior to this, *P. tritici-repentis* was described as a saprophyte. Secondly, *ToxA* has only been found in these two organisms to date. Finally, the nucleotide sequence of *SnToxA* exhibits greater diversity in its polypeptide sequence than that of *PtrToxA*. Taken together, this strongly suggests that *ToxA* was acquired by *P. tritici-repentis* before 1941 (Friesen *et al*. 2006; Stukenbrock and McDonald 2007). The identification of *SnToxA* in *S. nodorum* highlights the importance of genome sequencing in effector discovery (Hane *et al*. 2007). SnToxA and PtrToxA possess the same mode of action. Both effectors cause necrosis on wheat carrying *Tsn1* in a light-dependent manner (Manning and Ciuffetti 2005; Friesen *et al*. 2006).

The identification of *SnToxA* provided an opportunity to study the role of this gene in fungal virulence, as unlike *P. tritici-repentis*, *S. nodorum* is genetically tractable. Several lines of evidence have been published confirming that SnToxA interacts (directly or indirectly) with *Tsn1*. Firstly, *S. nodorum* strains lacking *SnToxA* were non-pathogenic on Tsn1 wheat varieties (Friesen *et al*. 2006). Secondly, protein extracts from *SnToxA*-expressing *S. nodorum* strains induced necrosis on *Tsn1* wheat, whilst extracts from *SntoxA* lines did not (Friesen *et al*. 2006). Lastly, transformation of an avirulent *ToxA*-deficient wild-type strain of *S. nodorum* with *PtrToxA* allowed the fungus to

become virulent and cause necrosis on *Tsn1* wheat lines (Friesen *et al*. 2006).

SnTox3 was the second necrotrophic effector gene identified in *S. nodorum*. SnTox3 was first reported as a partially purified protein that caused necrosis on wheat carrying the *Snn3* dominant sensitivity gene, which is located on the short arm of chromosome 5 (Friesen *et al*. 2008*b*; Liu *et al*. 2009). Gene knockout analysis of *SnTox3* indicated it to be a critical component in *S. nodorum* virulence on *Snn3* wheat. The introduction of *SnTox3* into an avirulent *SnTox3*-deficient *S. nodorum* wild-type strain allowed it to infect and cause necrosis on *Snn3* wheat varieties. Whilst detailed mechanistic studies have yet to be undertaken, SnTox3 appears to be functionally different to SnToxA. SnTox3 is cysteine-rich, a characteristic typically associated with several described biotrophic avirulence effectors (Van den Ackerveken *et al*. 1993; Catanzariti *et al*. 2006). Also, unlike SnToxA, SnTox3 does not require light to induce necrosis on *Snn3* wheat. This suggests a different mode of function compared with SnToxA. Gene expression analysis indicates that *SnTox3* is upregulated during the early stage of infection, coinciding with host penetration (Liu *et al*. 2009). *SnToxA* also showed a similar expression profile during infection (Ipcho and Oliver, unpubl. data).

These studies imply that these effectors function to disable host cells during the early stage of infection. Thus, the invading fungus will have a readily accessible nutrient supply during infection (Solomon *et al*. 2003).

S. nodorum also possesses at least three other proteinaceous necrotrophic effectors. These are SnTox1, SnTox2 and SnTox4. However, genes that code for these proteins have yet to be identified and therefore, the extent of their involvement in fungal virulence cannot be fully gauged (Liu *et al*. 2004; Friesen *et al*. 2007; Reddy *et al*. 2008; Abeysekara *et al*. 2009). The wheat genes that confer sensitivity to these effectors are *Snn1*, *Snn2* and *Snn4*, respectively. The use of molecular marker-based quantitative trait locus (QTL) analysis of various mapping populations of wheat has led to the identification of major QTLs in wheat chromosome arms 1BS (*Snn1*), 2DS (*Snn2*) and 1AS (*Snn4*) that accounted for up to 58%, 47% and 41% in disease variations, respectively (Friesen *et al*. 2008*a*; Abeysekara *et al*. 2009).

Effectors of other necrotrophic fungi

Proteinaceous effectors from other prominent necrotrophic fungi have also been recently identified. *Alternaria brassicae* (Berk.) Sacc. is a pathogen of the Brassicaceae. Evidence that this pathogen produces necrotrophic effectors was reported by Parada *et al*. (2008). Semi-purified protein fractions were shown to contain a 27.5 kDa protein, Abr-toxin, which is able to cause necrosis on cabbage (*Brassica oleracea* L.) and oilseed (*Brassica napus* L.). Abr-toxin caused no necrosis on the non-brassica tomato (*Lycopersicon esculentum* Mill.). Coinoculation of the Abr-toxin and an avirulent isolate of *A. alternata* resulted in infectious symptoms on the host leaf similar to *A. brassicae*. Partial protein sequencing revealed that the Abr-toxin possesses amino acid sequence similarities to the protease trypsin.

Pyrenophora teres f. sp. *teres* and f. sp. *maculata* Drechs. cause net-form net blotch and spot-form net blotch in barley (*Hordeum vulgare* L.), respectively. Using protein chromatographic techniques, Sarpeleh *et al*. (2007) demonstrated that both pathogens produce proteinaceous effectors that are between 20 and 100 kDa. These semi-purified effectors induce strong necrosis on a barley variety that is susceptible to both fungi, but caused a weak reaction in a resistant line of barley. Like PtrToxA and SnToxA, these effectors require light to cause necrosis on the host plant (Sarpeleh *et al*. 2008). The identity of these effectors from both *P. teres* subspecies is currently unknown.

Corynespora cassiicola (Berk. & Curtis) Wei, a serious pathogen of rubber trees (*Hevea brasiliensis* Müll. Arg.), produces a cysteine-rich necrotrophic effector called cassiicolin. The effector is able to cause necrosis on detached rubber tree leaves and on other host plants such as tobacco (*Nicotiana tabacum* L.) and soy (*Glycine max* (L.) Merr. (Barthe *et al*. 2007; de Lamotte *et al*. 2007). Although the deduced effector amino acid sequence did not show significant homology with other proteins, structural analysis indicates that the protein structure resembles trypsin-like inhibitors (Barthe *et al*. 2007).

The fungus *Rhynchosporium secalis* (Oudem.) Davis is the causal agent of barley scald. Several small cysteine-rich proteins designated as Nip1, -2 and -3 were identified in *R. secalis*, and these are capable of causing necrosis on a broad range of plants. Nip1 and 3 has been shown to stimulate barley plasma H^+ ATPase, which may be the likely cause of host tissue necrosis (Wevelsiep *et al*. 1991, 1993). Nip1 has recently been shown to bind to a single unidentified receptor that triggers the plant's defence response (van't Slot *et al*. 2007). In addition, Nip1 also functions as an avirulence effector on barley varieties that possess the uncloned *Rrs1* gene (Rohe *et al*. 1995). A total of 14 Nip1 forms were identified, three of which are associated with a gain in virulence on *Rrs1* barley (Schürch *et al*. 2004).

Conclusion

Necrotrophic fungi were, up until recently, considered as simplistic pathogens that rely on a plethora of non-host-specific mechanisms to storm the host. Recent seminal discoveries of host-selective necrotrophic effectors have revealed a new level of pathogenic complexity. These breakthroughs highlight that necrotrophic fungi possess the ability to disable their host selectively from within before an effective defence response can be mounted. The mode of action of these effectors is largely unknown, although studies on both ToxA proteins clearly demonstrate that the host metabolism is disabled before cell death. Hence, host-selective effectors are paramount for these fungi to live a necrotrophic lifestyle.

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