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 which 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 pathogens, *Pyrenophora tritici-repentis* and *Stagonospora nodorum*, have shed light upon how these effector proteins serve to disable the host from inside-out.
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. As with the avirulence (Avr) effectors of biotrophic fungal pathogens (reviewed in this issue), necrotrophic effectors share some common properties (Table 1). However, in contrast to the classical gene-for-gene hypothesis, where the interaction of avirulence effectors with host 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 (Figure 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 of *Cochliobolus victoriae* and AAL-toxin of *Alternaria alternata*, and these have been extensively reviewed elsewhere (Walton 1996; Wolpert *et al.* 2002). The purpose of this review will focus on discussing recent discoveries of proteinaceous necrotrophic effectors and their roles in virulence.

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

*Pyrenophora tritici-repentis* is the causal agent of tan spot (previously called either yellow spot or yellow leaf spot), a devastating disease of wheat. To date, two proteinaceous effectors of this necrotroph have been identified, *PtrToxA* and *PtrToxB* 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, which is present in approximately 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 and colleagues has 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 is
sufficient to render that isolate pathogenic on PtrToxA-sensitive wheat lines (Ciuffetti et al. 1997).

Wheat sensitivity to the PtrToxA effector is conditioned by *Tsn1*, a single gene present on the long arm of chromosome 5B (Anderson et al. 1999; Faris et al. 1996). 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 resistance (*R*) genes which are involved in defence against biotrophic pathogens (Martin et al. 2003).

Parallels can be drawn with the victorin effector of *Cochliobolus victoriae*, the causal agent of victoria blight disease of oats (Meehan and Murphy 1946). The *C. victoriae* susceptibility gene has been identified in *Arabidopsis thaliana* 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 arginy1-glycyl-aspartic (RGD) motif present at the surface of the PtrToxA protein (Zhang et
al. 1997), which is required for ToxA 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. 2005b). In animals, the RGD motif is involved in the binding of extracellular matrix proteins to transmembrane integrin proteins (D'Souza et al. 1991; Ruoslahti and Pierschbacher 1986). 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 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 (Baluska et al. 2003; Faik et al. 1998; Laboure et al. 1999; Nagpal and Quatrano 1999; Sun et al. 2000; Swatzell et al. 1999) 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), homologs of which have been found across several plant species (Manning et al. 2007; Sarma et al. 2005a). Although the precise function of ToxABP1 is unknown, it has been suggested to play a part in photosystem function and/or thylakoid formation (Keren et al. 2005; Wang et al. 2004). Indeed, PtrToxA treatment has been demonstrated to induce changes in photosystems I and II, 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 (Faris et al. 2010; Manning and Ciuffetti 2005).

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 up-regulated 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 Ptr which 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* (Martinez et al. 2001; Orolaza et al. 1995; Strelkov et al. 1999). Although not as prevalent as PtrToxA,PtrToxB has been found to be produced by a number of isolates around the world (Ali and Francl 2003; Friesen and Faris 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 (Friesen and Faris 2004; Strelkov et al. 1999). 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* is the causal agent of stagonospora (previously Septoria) nodorum blotch (SNB) of wheat (Solomon et al. 2006). Evidence of necrotrophic effectors produced by *S. nodorum* was first reported by Keller and colleagues (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 11 kb genomic region 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* prior to 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 (Friesen et al. 2006; Manning and Ciuffetti 2005).

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 wildtype strain of *S. nodorum* with PtrToxA allow the fungus to become virulent and cause necrosis on wheat lines harbouring *Tsn1* (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 causes necrosis on wheat carrying the *Snn3* dominant sensitivity gene which is located on the short arm of chromosome 5 (Friesen et al. 2008b; 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 Tox3-deficient *S. nodorum* wildtype 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 (Catanzariti et al. 2006; Van den Ackerveken et al. 1993). Also, unlike ToxA, SnTox3 does not require light to induce necrosis on *Snn3* wheat. This suggests a different mode of function compared to SnToxA. Gene expression analysis indicates that SnTox3 is up-regulated during the early stage of infection coinciding with host penetration (Liu et al. 2009). SnToxA also showed a similar expression profile during infection (unpublished 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 (Abeysekara et al. 2009; Friesen et al. 2007; Liu et al. 2004; Reddy et al. 2008). 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 wheat mapping populations 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 (Abeysekara et al. 2009; Friesen et al. 2008a).

**Effectors of other necrotrophic fungi**

Proteinaceous effectors from other prominent necrotrophic fungi have also been recently identified. *Alternaria brassicae* 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, able to cause necrosis on cabbage and oilseed. It caused no necrosis on the non-brassica, tomato. Co-inoculation 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* cause net-form net blotch and spot-form net blotch of barley, respectively. Using protein chromatographic techniques, Sarpeleh et al. (2007) demonstrated that both pathogens produce proteinaceous effectors that are between 20 to 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 ToxA, 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*, a serious pathogen of rubber trees, produces a cysteine-rich necrotrophic effector called cassiicolin. The effector is able to cause necrosis on detached rubber tree leaves and other host plants such as tobacco and soy (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* 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; Wevelsiep et al. 1993). Nip1 has recently been shown to bind to a single unidentified receptor that triggers the plant defense 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 (Schurch 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 selectively disable their host from within, before an effective defence response can be mounted. The mode of action of these effectors is largely unknown, although studies on ToxA clearly demonstrate that host metabolism is disabled prior to cell death. Hence, host-selective effectors are paramount for these fungi to live a necrotrophic lifestyle.
Table 1. Properties of proteinaceous avirulence and necrotrophic effectors.

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 the absence or altered R (resistance) receptor (B) or Avr effector (C). Necrotrophic effectors function in an inverse manner (D to F). A successful infection will only occur during effector recognition (D). During a non-recognition event, no disease will result due to the absence 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.
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<table>
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<tr>
<th>Characteristic</th>
<th>Avirulence effector</th>
<th>Necrotrophic effector</th>
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<tbody>
<tr>
<td>Relative small size?</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Secreted</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Expression in planta</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Receptor type(^)</td>
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<tr>
<td>Cysteine-rich(*)</td>
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<td>Some</td>
</tr>
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<td>Compatible host response</td>
<td>No disease</td>
<td>Disease</td>
</tr>
<tr>
<td>Fungal lifestyle</td>
<td>Biotrophic/hemibiotrophic</td>
<td>Necrotrophic</td>
</tr>
<tr>
<td>Function/homology</td>
<td>Diverse</td>
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\(^#\) Under 30 kDa.

\(^\) NBS-LRR denotes nucleotide-binding site leucine-rich repeat protein.

\(*\) Greater than four cysteines per mature polypeptide.
Figure 1

(A) Recognition
- Avr
  - Fungus
  - Plant cell
  - NO DISEASE

(B) Non-recognition
- Avr
  - Fungus
  - Plant cell
  - DISEASE

(C) Non-recognition
- R receptor
  - Fungus
  - Plant cell
  - DISEASE

(D) Necrotrophic effector (HST)
- HST
  - Fungus
  - Plant cell
  - DISEASE

(E) Fungus
- HST
  - Fungus
  - Plant cell
  - NO DISEASE

(F) Fungus
- Sensitivity receptor
  - Fungus
  - Plant cell
  - NO DISEASE