REVIEW ARTICLE

Phytotoxic secondary metabolites and peptides produced by plant pathogenic *Dothideomycete* fungi

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Abstract

Many necrotrophic plant pathogenic fungi belonging to the class of *Dothideomycetes* produce phytotoxic metabolites and peptides that are usually required for pathogenicity. Phytotoxins that affect a broad range of plant species are known as non-host-specific toxins (non-HSTs), whereas HSTs affect only a particular plant species or more often genotypes of that species. For pathogens producing HSTs, pathogenicity and host specificity are largely defined by the ability to produce the toxin, while plant susceptibility is dependent on the presence of the toxin target. Non-HSTs are not the main determinants of pathogenicity but contribute to virulence of the producing pathogen. *Dothideomycetes* are remarkable for the production of toxins, particularly HSTs because they are the only fungal species known so far to produce them. The synthesis, regulation, and mechanisms of action of the most important HSTs and non-HSTs will be discussed. Studies on the mode of action of HSTs have highlighted the induction of programed cell death (PCD) as an important mechanism. We discuss HST-induced PCD and the plant hypersensitive response upon recognition of avirulence factors that share common pathways. In this respect, although nucleotide-binding-site-leucine-rich repeat types of resistance proteins mediate resistance against biotrophs, they can also contribute to susceptibility toward necrotrophs.

Introduction

Plant pathogenic fungi are commonly divided into biotrophic, hemi-biotrophic, and necrotrophic pathogens. Biotrophic pathogens thrive on living host tissues and often secrete effectors to suppress the host immune system, thus enabling them to cause disease (Stergiopoulos & de Wit, 2009). In contrast, necrotrophic fungal pathogens thrive on dead host tissue that has become necrotized by secreted cell wall degrading enzymes, phytotoxic secondary metabolites (SMs), peptides, and reactive oxygen species (ROS; Horbach *et al.*, 2011). Finally, hemi-biotrophic pathogens initially establish a biotrophic relationship with their host and produce toxins only at later stages of the infection to kill the host cells and enable the pathogen to complete its life cycle on the dead tissue (Horbach *et al.*, 2011). Secreted phytotoxins contribute to virulence or pathogenicity by disrupting host cells and inducing the release of nutrients to facilitate colonization of host tissues (Wolpert *et al.*, 2002; Berestetskiy, 2008). Although the division of pathogenic fungi in these three lifestyles provides a general impression about their infection and interaction with their host, the distinction between biotrophs and hemi-biotrophs is often less clear.

Classification of toxins has been traditionally based on the spectrum of their biological activity rather than on their chemical properties or structural characteristics. In this way, two major classes are distinguished as follows: host-specific toxins (HSTs) and non-HSTs (Wolpert *et al.*, 2002; Berestetskiy, 2008).

HSTs are biologically active only against a particular plant species and essentially determine the host range of the producing pathogen (Wolpert *et al.*, 2002). Often, not all genotypes of a host plant species are sensitive to the toxin and similarly not all isolates of a pathogen species produce the toxin. Thus, the genetic basis of interaction

FEMS MICROBIOLOGY REVIEWS

between HSTs and plant genotypes resembles that of race-specific effectors produced by (hemi-)biotrophic plant pathogens and cognate resistant genes. The difference is that, unlike resistant genes, the presence of a HST target allele in a specific plant genotype confers susceptibility to the pathogen rather than resistance. This concept is generally known as the inverse gene-for-gene model, as opposed to the classical gene-for-gene model that holds for (hemi-)biotrophic pathogen-host interactions (Oliver & Solomon, 2010). Nearly all HSTs identified so far are produced by necrotrophic pathogens of the order of *Pleosporales* within the class of *Dothideomycetes*. Treatment of the plant genotype with the HST alone invokes the same disease symptoms as the pathogen itself, while disruption of the pathogen's ability to produce the toxin results in complete or severe loss of pathogenicity on the plant genotype (Wolpert *et al.*, 2002).

Unlike HSTs, non-HSTs are biologically active against a broad spectrum of plant species (Berestetskiy, 2008) and are neither the sole determinants of pathogenicity nor define host specificity. Instead, they mainly contribute to virulence of the producing pathogen. A wide variety of non-HSTs implicated in disease development have been characterized from different *Dothideomycete* plant pathogenic fungi and they act on different physiological processes common in plants, including energy production (tentoxin: *Alternaria* spp.), lipid biosynthesis (cyperin: *Ascochyta cypericola*), actin polymerization (cytochalasins: many fungal species), production of ROS (cercosporins: *Cercospora* spp.) and various others (Berestetskiy, 2008).

This review focuses on HSTs and non-HSTs produced by species of *Dothideomycetes*, the largest and taxonomically most diverse class of *Ascomycete* fungi (Hane *et al.*, 2011). The vast majority of HSTs and non-HSTs are low molecular weight SMs (Collemare & Lebrun, 2011) and different species of *Dothideomycetes* produce different profiles of SMs that are dispensable for growth. Their production is highly dependent on the physiological or morphological stage of the fungus and the conditions under which it is grown. SM non-HSTs and HSTs produced by fungi during infection of the host can be important determinants of pathogenicity or virulence. Based on their chemical structure, SMs are mostly classified as polyketides, nonribosomal peptides, alkaloids, terpenes, or metabolites of mixed biosynthetic origin (Collemare & Lebrun, 2011). Some HSTs are ribosome-produced peptides, as reported for the fungi *Pyrenophora tritici-repentis* (*Ptr*) and *Stagonospora nodorum* (Friesen *et al.*, 2008a, b).

SM HSTs produced by *Dothideomycetes*

SM HSTs are mainly produced by species of *Cochliobolus* and *Alternaria*. Those produced by *Alternaria* species are covered in another review to be published in this journal (Tsuge, *et al.* 2012; this issue). Several pathogenic species and races of *Cochliobolus* (anamorph state *Bipolaris* or *Curvularia*) are known to produce an array of structurally diverse HSTs that are primary virulence factors (Table 1; Fig. 1). HST producing species of *Cochliobolus* are so far exclusively pathogens of grasses. For most of these species the ability to produce the HST as well as sensitivity of host plants to the toxin are controlled by single Mendelian genes or genetic loci. However, the mode of action and the way HSTs manipulate the host to enable infections differ significantly from one producing species to another. On the other hand resistance toward the HST is conferred either by the lack of a target site of the toxin or by the ability to detoxify the HST. Here, we summarize what is known about the remarkable complexity and role during plant–microbe interactions of the major HSTs produced by species of *Cochliobolus*.

HC-toxin is a cyclic tetrapeptide produced by *C. carborum*, a fungus causing Northern corn leaf spot and ear rot of maize (Walton, 2006). The disease emerged in the first half of the 20th century and HC-toxin isolated from *Cochliobolus carbonum* was identified as a major determinant of host specificity and virulence in the 1960s. HC-toxin is only produced by race 1 isolates of the fungus, which generally cause larger lesions on susceptible maize cultivars compared with the HC-toxin-deficient races 2 and 3 (Walton *et al.*, 1997). HC-toxin is an important virulence factor and a major determinant of the disease on maize. Consistent with the emergence of the disease, *C. carbonum* race 1 isolates were only reported after the introduction of new corn varieties in the late 1930s that contained a susceptibility locus to the HC-toxin (Ullstrup & Brunson, 1947). The structure of HC-toxin is cyclo (D-Pro-L-Ala-D-Ala-L-Aeo), where Aeo stands for 2aminoepoxyoxo decanoic acid, a side chain with a carbonyl function that is indispensable for toxicity on susceptible hosts (Fig. 1; Walton *et al.*, 1982). The mode of action of HC-toxin is based on inhibition of histone-deacetylases (HDACs) that are generally associated with repression of gene expression and consequently, their inhibition leads to H3 and H4 histone-hyperacetylation in maize and changes in transcript levels (Brosch *et al.*, 1995). Thus, epigenetic transcriptional modification in presumably host defense genes is thought to be the primary role of HC-toxin in disease (Brosch *et al.*, 1995). Host susceptibility to the toxin on the other hand is conferred by the lack of production of a carbonyl HC-toxin reductase (HCTR), an enzyme that detoxifies HC-toxin through chemical reduction of a side carbonyl group (Meeley & Walton, 1991; Walton, 2006). The NADPH-dependent HCTR in maize is encoded by the dominant *Hm1* nuclear gene, the first plant resistance gene ever to

Table 1. Biological activities of SM and proteinaceous HSTs produced by species of *Dothideomycetes*

Toxin	Species	Chemical structure	Mode of action	Plant target
HC-toxin	*Cochliobolus carbonum*	Cyclic tetrapeptide	Suppression of host defenses	HDACs
T-toxin	*Cochliobolus heterostrophus*	Linear polyketide	Disruption of mitochondrial activity	URF13: 13-kDa mitochondrial protein
PM-toxin	*Mycosphaerella zeae-maydis*	Linear polyketide	Disruption of mitochondrial activity	URF13: 13-kDa mitochondrial protein
Victorin	*Cochliobolus victoriae*	Cyclic chlorinated pentapeptide	Induction of PCD	LOV1: CC-NB-LRR disease resistance protein
HS-toxin	*Bipolaris sacchari*	Sesquiterpene glucoside	Depolarization of plasma membrane	Unknown
Phomalide	*Leptosphaeria maculans*	Cyclic pentadepsipeptide	Unknown	Unknown
Depsilairdin	*Leptosphaeria maculans* Laird 2 and Mayfair 2	Sesquiterpenic depsipeptide	Unknown	Unknown
Maculansins A and B	*Leptosphaeria maculans*	D-mannitol derived SM	Unknown	Unknown
PC-toxin	*Periconia circinata*	Peptidyl chlorinated polyketide	Induction of PCD	Unknown
SV-toxins I and II	*Stemphylium vesicarium*	Unknown	Induction of PCD	Unknown
PtrToxA	*Pyrenophora tritici-repentis*	13.2-kDa protein	*Tsn1*-mediated induction of PCD	Chloroplasts, ToxABP1
PtrToxB	*Pyrenophora tritici-repentis*	6.5-kDa protein	Induction of PCD	Probably chloroplasts
SnToxA	*Stagonospora nodorum*	13.2-kDa protein	*Tsn1*-mediated induction of PCD	Chloroplasts, ToxABP1
SnTox1	*Stagonospora nodorum*	10.3-kDa protein	*Snn1*-mediated induction of PCD	Probably chloroplasts
SnTox2	*Stagonospora nodorum*	7–10-kDa protein	*Snn2*-mediated induction of PCD	Probably chloroplasts
SnTox3	*Stagonospora nodorum*	25.8-kDa protein	*Snn3*-mediated induction of PCD	Unknown
SnTox4	*Stagonospora nodorum*	10.3-kDa protein	*Snn4*-mediated induction of PCD	Probably chloroplasts

be cloned (Johal & Briggs, 1992). In the absence of *Hm1*, a second dominant allele at an unlinked locus, *Hm2*, can also confer resistance to the fungus, albeit at intermediate levels as compared to *Hm1* and only in adult plants. Molecular cloning and sequencing of both genes has indicated that *Hm2* is a duplication of *Hm1* (Johal & Briggs, 1992; Multani *et al.*, 1998). *Hm* homologs appear to be evolutionary conserved in all monocots, suggesting that *Hm*-mediated resistance to *C. carborum* is of ancient origin and probably wide-spread in grasses (Han *et al.*, 1997). However, two molecular events in maize, a transposon insertion in *Hm1* and a deletion in *Hm2*, and subsequent inbreeding for hybrid seed production led to the breakdown of the natural mechanism of resistance in this crop and the emergence of disease (Multani *et al.*, 1998). On the other hand, *C. carbonum* seems to employ two different mechanisms of self-protection against the HC-toxin. The first one is presumably based on active export of the toxin by the TOXA transporter, a member of the major facilitator superfamily of transporters (Pitkin *et al.*, 1996). Additionally, *C. carbonum* displays two HDAC activities, one of them being toxin-resistant (Brosch *et al.*, 2001).

T-toxin, formerly known as HMT-toxin or BMT-toxin, is synthesized by the necrotrophic fungus *Cochliobolus heterostrophus*, the causal agent of southern corn leaf blight (SCLB) in maize. Production of T-toxin is associated with high aggressiveness of the pathogen on maize cultivars that carry the '*Texas male sterile cytoplasm*' (*T-cms*). Indeed, race T isolates that produce T-toxin are significantly more virulent on *T-cms* genotypes than the nontoxin-producing race O isolates (Tegtmeier *et al.*, 1982). However, although it enhances virulence, T-toxin is not required for pathogenicity of *C. heterostrophus* race T strains on *T-cms* maize genotypes as toxin-deficient disruption mutants of the fungus are still able to cause

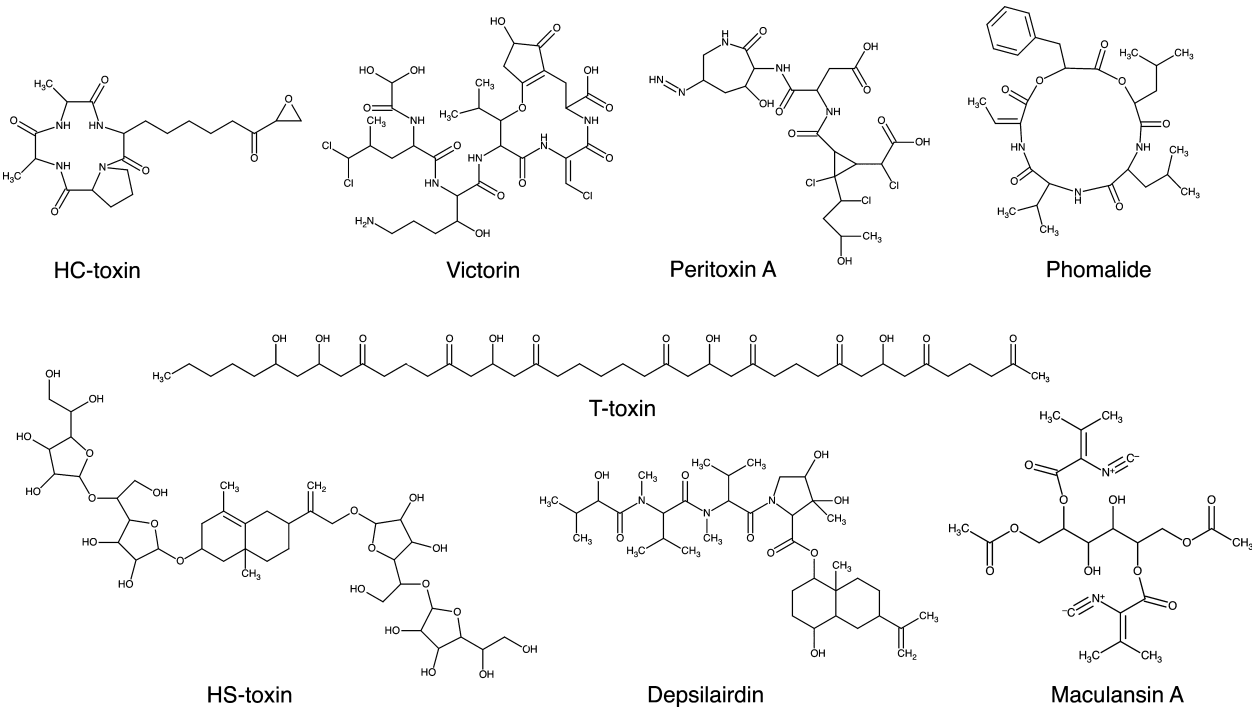

Fig. 1. Structures of SM HSTs produced by fungal species of *Dothideomycetes.*

disease symptoms (Walton, 1996). The existence of *C. heterostrophus* race T isolates became widely acknowledged in the early 1970s, when an SCLB epidemic ravaged cornfields across the US, causing what is considered by many as one of the most economically devastating diseases of the 20th century (Ullstrup, 1972). This epidemic was triggered by the vast monoculture of susceptible maize lines, after the introduction in the 1950s and 1960s of *T-cms* parental lines for the production of hybrid lines that did not necessitate hand or mechanical emasculation (Ullstrup, 1972). T-toxin is a linear polyketide containing between 35 and 49 carbons, although found predominately as a C_{41} derivative (Fig. 1; Kono *et al.*, 1985). Its mode of action is based on conformational changes induced upon binding of the toxin on the gene product of *T-urf13*, the Texas cytoplasmic male sterility gene. *T-urf13* is located on the mitochondrial chromosome of T-cytoplasm genotypes and encodes a 13 kDa oligomeric protein (URF13) that is assembled in the inner mitochondrial plasma membrane (Wise *et al.*, 1987). URF13 causes premature degeneration of the mitochondria-rich tapetal cell layer of anthers at an early microspore stage, resulting in pollen abortion. Direct binding of T-toxin to URF13 induces pore formation and rapid permeabilization of the inner mitochondrial membrane that disrupts regular function and elicits a number of responses, including uncoupling of oxidative phosphorylation, leakage of small molecules, breakdown of electrochemical gradient and ATP-production, and membrane swelling (Levings *et al.*, 1995). These T-toxin-dependent effects are also observed when URF13 is expressed in *Escherichia coli*, yeast, tobacco, and insects). Although the mode of action of T-toxin and related polyketides is solved, little is known about the function of URF13 in mitochondria, which originates from a series of recombination events involving other mitochondrial genes (Dewey *et al.*, 1986). This origin raises the question of URF13 occurrence in nature, and consequently of the function of T-toxin for *C. heterostrophus*. Indeed, distribution of SM biosynthetic pathways is discontinuous among fungi, suggesting that maintenance of SM production in a given species is owing to evolutionary constraints. It is possible that T-toxin may play another role in the biology of *C. heterostrophus* rather than solely being a HST on maize. PM-toxin is an analog of T-toxin that is produced by the yellow leaf blight pathogen of corn *Mycosphaerella zeae-maydis* (recently renamed *Pyronellaea zeae-maydis* (Aveskamp *et al.*, 2010). This fungus is pathogenic on *T-cms* corn mainly owing to the production PM-toxin. PM-toxin is a polyketide with a structure similar and its role in the pathogenicity of only *M. zeae-maydis* seems to be more important than T-toxin for *C. heterostrophus* as mutants deficient for PM-toxin production are no longer pathogenic on *T-cms* maize genotypes (Yun *et al.*, 1998).

Victorin is a cyclic pentapeptide (Fig. 1) produced by the fungus *Cochliobolus victoriae*, the causal agent of the

Victoria blight of oats. During the 1940s an epidemic swept across oat fields in the US that were planted with lines containing the 'Victoria-type' of resistance toward the crown rust fungus *Puccinia coronata* f. sp. *avenae* (Meehan & Murphy, 1946; Meehan, 1947). Resistance to crown rust is conferred by the dominant *Pc-2* gene, a desirable trait that was extensively bred in oats and lead to the Victoria blight epidemic, as *Pc-2* containing oats turned out to be susceptible to the new disease. Conversely, pathogenicity of *C. victoriae* on *Pc-2* lines depended largely on the production of victorin (Macko *et al.*, 1985). Victorin is a HST required for pathogenicity because only oat genotypes that are sensitive to victorin are susceptible to *C. victoriae*, treatment with victorin alone is able to reproduce disease symptoms on sensitive plants (Navarre & Wolpert, 1999; Lorang *et al.*, 2004). Sensitivity of oat to victorin is controlled by a single dominant allele at the *Vb* locus. However, extensive genetic and mutational analysis failed to separate this locus from *Pc-2* that confers resistance to rust, suggesting that *Vb* and *Pc-2* were the same or closely linked loci (Mayama *et al.*, 1995). This implied an unexpected relation between disease resistance genes that at the same time were conferring susceptibility to a necrotrophic pathogen. Nevertheless, physiological studies on the mode of action of victorin supported this connection. Indeed, in sensitive plants victorin triggers both the induction of host defense responses as well as responses typical of a programed cell death (PCD; Navarre & Wolpert, 1999). The mechanism underlying the induction of PCD by victorin is unclear owing to contradictory reports that suggest the target of victorin to be either in mitochondria, where it would inhibit two components of the glycine decarboxylase complex (GDC; Navarre & Wolpert, 1995), or at the plasma membrane, as the PCD occurs before victorin enters the cell and is independent of GDC binding (Tada *et al.*, 2005). Understanding the mechanism of *C. victoriae* susceptibility was considerably advanced by the molecular dissection of victorin susceptibility in *Arabidopsis thaliana*, which led to the identification on chromosome 1 of the *Locus Orchestrating Victorin effects 1*, or *LOV1* (Lorang *et al.*, 2007). *LOV1* encodes a coiled-coil-nucleotide-binding-site-leucine-rich repeat (CC-NBS-LRR) protein that is highly similar to intracellular resistance proteins of the RPP8 family. Although perception of victorin by LOV1 induces several disease resistance-associated responses, including rapid induction of the pathogenicity-related gene *PR-1* and production of the phytoalexin camalexin, LOV1-mediated susceptibility does not require salicylic acid, jasmonic acid, or ethylene-mediated signaling pathways (Lorang *et al.*, 2007). In a similar way, RAR1, SGT1b and HSP90, which are required by many resistance genes for the expression of complete resistance, do not contribute to LOV1-mediated susceptibility (Lorang *et al.*, 2007). Interestingly, a thioredoxin is required for victorin sensitivity (Sweat & Wolpert, 2007), which is reminiscent of the involvement of the CITRX thioredoxin in tomato Cf-dependent resistance responses (Rivas *et al.*, 2004). These results suggest that victorin may actually target a typical resistance protein that is normally involved in the recognition of a naturally occurring pathogen of *A. thaliana* (Sweat *et al.*, 2008). Such a target may also exist in oat, the natural host of *C. victoriae*, and could be the resistance gene located at the *Pc-2* locus. In that case, the *Vb/Pc-2* locus might be the first example of a gene involved in resistance against a biotroph (*P. coronata* f. sp. *avenae*)and susceptibility toward a necrotroph (*C. victoriae*).

HS-toxin, also known as helminthosporoside, is produced by *Bipolaris sacchari* (also known as *H. saccahri* or *Drechslera sacchari*), the causal agent of the eyespot disease of sugarcane. HS-toxin consists of four β-1,5-linked galactofuranose units linked with the asymmetric sesquiterpene core ($C_{15}H_{24}O_2$) at carbon positions 2 and 13 (Fig. 1; Macko *et al.*, 1983). HS-toxin causes lesions and electrolyte leakage only on sugarcane cultivars susceptible to *B. sacchari* and thus can be considered as a HST (Schroter *et al.*, 1985). Membrane depolarization is also observed as a result of its activity, an effect that is reversible by increasing temperature at 30 °C or higher (Schroter *et al.*, 1985). However, although a good correlation between sensitivity to the toxin and susceptibility to the pathogen is in general observed, other factors might be contributing to the disease as well, because in some cases, disease severity did not correlate with sensitivity to the toxin (Bournival *et al.*, 1994). In addition to HS-toxin, *B. sacchari* can produce lower molecular weight toxin-analogs (toxoids) that lack one or more of the galactose residues and act antagonistically to the HS-toxin, thus protecting sensitive tissues against the toxin (Livingston & Scheffer, 1984). The biological relevance of the production of these analogs by the fungus is not understood yet, but their production could suggest that they interact or compete for the yet to be identified HS-toxin receptor.

The plant pathogenic fungus *Leptosphaeria maculans* (anamorph *Phoma lingam*) causes blackleg disease, one of the most devastating diseases of the oilseed crops canola and rapeseed (Howlett *et al.*, 2001). The fungus is known to produce an array of SMs under diverse conditions and there is some evidence that a few of them might act as HSTs (Table 1; Fig. 1; Pedras & Yu, 2009). Phomalide was originally isolated from liquid minimal-medium cultures of *L. maculans* that were grown for 24–60 h but the toxin is known to be produced by the fungus during infection of susceptible hosts as well, suggesting a role during pathogenesis (Elliott *et al.*, 2007). The toxin causes

reddish, chlorotic and necrotic lesions on canola leaves that closely resemble those induced by the pathogen. These symptoms are only present on susceptible canola and rapeseed and no damage is observed on resistant *Brassica* species, such as brown mustard and white mustard, even when the toxin is applied at high concentrations (10^{-4} M; Ward *et al.*, 1999). The strong correlation between susceptibility to the toxin and pathogenic range of the fungus suggests that phomalide is an important determinant of host specificity and virulence. The structure of phomalide has been recently resolved as a rare cyclic depsipeptide with three α-amino acids and two α-hydroxy acids (Fig. 1; Siodlak *et al.*, 2011). Interestingly, the structural analog isophomalide that contains (*Z*)-dehydrobutyrine shows no phytotoxic activity against *Brassica* species, while the saturated dihydrophomalide causes chlorotic symptoms on the resistant brown mustard but not on canola, indicating that both the presence and configuration of the (*E*)-double bond is crucial for selective phytotoxicity (Siodlak *et al.*, 2011). Another depsipeptide produced by *L. maculans* is depsilairdin. The toxin is isolated from Laird 2 and Mayfair 2, two *L. maculans* isolates that are avirulent on canola but virulent on brown mustard, thus exhibiting reverse pathogenicity as compared to the rest of the isolates of the fungus (Pedras *et al.*, 2004). Treatment of brown mustard leaves with the toxin triggers the formation of chlorotic and necrotic spots, symptoms that resemble those caused by infections with the pathogen (Pedras *et al.*, 2004). The chemical structure of depsilairdin has been resolved and it is shown to be a sesquiterpenic depsipeptide that contains a (2S,3S,4S)-3,4-dihydroxy-3-methylprolyl residue (Dhmp), an amino acid that has never been described before (Fig. 1; Pedras *et al.*, 2004). Recently, the complete synthesis of depsilairdin and three depsilairdin analogs, in which the Dhmp residue was replaced with L-proline and cis- and trans-4-hydroxy-L-proline has been achieved. Analysis of phytotoxicities showed that the three analogs were no longer toxic to both brown mustard and canola, suggesting that the presence of Dhmp in the chemical structure of depsilairdin is required for its biological activity and host-selectivity to brown mustard (Ward & Pardeshi, 2010). A new set of selective phytotoxins, named maculansins A and B have also been recently isolated from virulent isolates of *L. maculans* (Pedras & Chumala, 2011). Notably, both maculansin A and B show higher toxicity on leaves of resistant brownmustard than on leaves of susceptible canola. Given the fact that Laird 2 and Mayfair 2 are pathogenic on brown mustard, it will be interesting to examine whether these two isolates can also produce maculansins A and B and whether these two metabolites contribute to virulence on brown mustard or relate to a host jump. Structurally, these toxins are derivatives of mannitol containing the unusual chromophore 2-isocyano-3-methyl-2-butenoyl (Fig. 1). Together with brassicicolin, a host selective phytotoxic metabolite produced by *Alternaria brassicicola*, these two metabolites seem to constitute a unique group of D-mannitol derived fungal metabolites with phytotoxic activities (Pedras & Chumala, 2011).

Less common pathogenic fungi also produce toxic SMs that might be determinants of a host range. PC-toxin, also known as peritoxin, is produced by the necrotrophic soil-borne fungus *Periconia circinata*, which causes milo disease, a root and crown rot of sorghum (Macko *et al.*, 1992; Dunkle & Macko, 1995). Peritoxins (Fig. 1) are hybrid molecules consisting of a peptide and a chlorinated polyketide with equal toxicities and which are known as peritoxins A and B, respectively (Macko *et al.*, 1992). Treatment of sorghum seedlings with peritoxin induces visual symptoms of disease only in susceptible genotypes (Dunkle & Macko, 1995). In addition, it causes a number of cytological and developmental alterations, including inhibition of mitosis, electrolyte leakage, vacuolar expansion, defects in secretory activity and endomembrane flow, dispersion of heterochromatin in the nuclei, autolysis and other hallmarks of apoptosis (Dunkle & Macko, 1995). Taken together, these observations suggest that the mode of action of peritoxin may be controlled by a disease resistance gene. Indeed, susceptibility to the pathogen and sensitivity to peritoxin in sorghum are both controlled by the single gene *Pc-B* that encodes a NBS–LRR type of resistance protein (Nagy & Bennetzen, 2008). To confirm the dual role of this type of proteins in resistance and susceptibility, it would be interesting to identify the biotrophic pathogen that is recognized by Pc-B. SV-toxins I and II are produced by *Stemphylium vesicarium*, the causal agent of brown spot of European pear, a disease of economic importance in Mediterranean pear-producing areas (Singh *et al.*, 1999). Despite the broad host range of *S. vesicarium*, biological studies have shown that gain of pathogenicity on pears is owing to the ability of the fungus to produce SV-toxins, suggesting a parasitic differentiation in the species owing to the acquisition of HSTs. Indeed, sensitivity of pear cultivars to the pathogen is directly correlated to their susceptibility toward the two toxins. SV-toxins selectively induce vein necrosis on susceptible pear cultivars, with SV-toxin I exhibiting almost 10 × higher toxicity than SV-toxin II (Singh *et al.*, 1999). On the cellular level, SV toxins induce electrolyte leakage from susceptible tissues and cause plasmalemmal invagination at both ends of plasmodesmata and other plasma membrane disorders that resemble those induced by the *Alternaria* AK, AF and ACT HSTs (Singh *et al.*, 2000). These effects suggest the target site of SV-toxins to be located on the plasma membrane near plasmodesmata.

However, as this target still remains elusive, it is not yet known whether the observed plasma membrane disorders are caused by direct binding of the SV-toxin to the membrane, or indirectly (Singh *et al.*, 2000).

Biosynthesis and regulation of SM HSTs

The biosynthesis of SMs involves many enzymatic steps. In fungi, the genes encoding proteins that catalyze these different steps are frequently organized in clusters and thus are located at the same locus and are often co-regulated (Keller & Hohn, 1997). Such SM gene clusters usually contain a key enzyme [e.g. a polyketide synthase (PKS), a nonribosomal peptide synthetase (NRPS), a terpene cyclase (TC), or a dimethylallyl tryptophan synthase] responsible for the biosynthesis of the first intermediate. In addition, they may also contain genes encoding additional enzymes, transporters and transcription factors (Keller *et al.*, 2005). Most studies to understand the biosynthesis of SM HSTs have so far focused on identifying the corresponding gene clusters. These efforts however, have only been successful for T-toxin and HC-toxin. There is no doubt that the availability of more fungal genomes will allow the characterization of additional SM biosynthetic pathways.

The biosynthesis of T-toxin in *C. heterostrophus* is controlled by the two unlinked loci *TOX1A* and *TOX1B*. These two loci behave like a single genetic locus because they are located on chromosomes that form a four-armed linkage group owing to a reciprocal translocation (Kodama *et al.*, 1999). The sequence complexity of these loci generated by extensive genetic rearrangements has so far hampered the identification of genes involved in T-toxin production. However, the availability of the genome sequence of the fungus accelerated their delineation, and a total of nine genes (*PKS1*, *PKS2*, *LAM1*, *OXI1* and *TOX9* at the *TOX1A* locus; *DEC1*, *RED1*, *RED2* and *RED3*, at the *TOX1B* locus) are now implicated in the production of T-toxin (Inderbitzin *et al.*, 2010). All genes at the *TOX1A* and *TOX1B loci* are embedded in A/T rich regions and are flanked by multiple repeats (Inderbitzin *et al.*, 2010). *PKS1* and *PKS2* encode fully reducing iterative type I PKSs that retain all the typical domains of this enzymatic family (Yang *et al.*, 1996; Baker *et al.*, 2006). However, the contribution of each PKS to the synthesis of the T-toxin polyketide backbone is still unknown. It is also unclear how the two polyketides are linked with each other, as they involve a unique carbon-carbon bond (Baker *et al.*, 2006). Further on, *DEC1* encodes an acetoacetate decarboxylase and its disruption completely abolishes the production of T-toxin (Rose *et al.*, 2002). This enzyme is likely responsible for the decarboxylation of the penultimate precursors, which explains that all polyketides of the T-toxin family terminate with no carboxyl function. Single mutants of the remaining six genes show decreased T-toxin production, while double and triple mutants completely lose their ability to produce the toxin (Inderbitzin *et al.*, 2010). These genes encode five dehydrogenases and one protein with unknown function (*TOX9*). The contribution of these accessory enzymes in T-toxin biosynthesis is still unknown. The transcript regulation of the *TOX1* genes has not been studied yet, but production of T-toxin *in vitro* and during maize infection indicates that they are all expressed under such conditions. Yet, a transcription factor that could regulate the expression of these genes has not been identified at none of the two *TOX1* loci. Thus, it is possible that genes located on other scaffolds are involved in T-toxin production and regulation. Recently, T-toxin production was shown to be under the epigenetic control of LaeA and VeA orthologs in *C. heterostrophus,* which regulate sporulation and secondary metabolism in *Aspergillus nidulans* (Wu *et al.*, 2012). Indeed, a positive correlation between expression of *TOX1* genes and consequently production of T-toxin is observed in the dark, in mutants that overexpress or are depleted of LaeA and VeA activity, respectively (Wu *et al.*, 2012). These are the only regulators of T-toxin identified so far.

PM-toxin produced by *M. zeae-maydis* is structurally related to T-toxin and involves a gene cluster that contains homologs of *TOX1* genes (*PKS1*, *RED1* and *RED2*; Inderbitzin *et al.*, 2010). Although the three genes are required for PM-toxin biosynthesis in *M. zeae-maydis*, this gene cluster does not provide any additional information on the biosynthesis of T-toxin.

Biosynthesis of HC-toxin is governed by the *TOX2* locus in *C. carbonum*. The locus spreads over a 600-kb genomic region and contains at least seven genes in multiple copies (*HTS1*, *TOXA*, *TOXC*, *TOXD*, *TOXE*, *TOXF* and *TOXG*; Ahn *et al.*, 2002). Like the *TOX1* locus, the *TOX2* locus appears to be subject to rearrangements, likely caused by the activity of transposable elements (Panaccione *et al.*, 1996). Apart of *TOXD,* all other genes present in this locus are shown to be involved in the biosynthesis, export or regulation of HC-toxin production, and consequently are all required for pathogenicity on maize. *HTS1* is considered the key gene in the biosynthesis of the HC-toxin. The gene encodes a 570-kDa NRPS, composed of four modules and each responsible for the addition of a single aminoacid in the peptide product (D-Pro, L-Ala, D-Ala and L-Aeo, respectively; Panaccione *et al.*, 1992). The modular order in HTS1 fully corresponds to the amino acid order in the final peptide product. The epimerization of L-Pro to D-Pro is catalyzed by an epimerase domain present within the first module of HTS1. The second D-amino acid is epimerized by another enzyme, an alanine racemase, encoded by *TOXG* (Cheng

& Walton, 2000). However, this amino acid is not required for HC-toxin activity as *Δ to xg* mutants are still able to produce a minor form of HC-toxin, in which alanine is replaced by glycine (Cheng & Walton, 2000). This is consistent with the finding that HC-toxin activity is owing to both the terminal epoxide and the vicinal carbonyl group of Aeo (Brosch *et al.*, 1995). The biosynthesis of the Aeo chain is an important step in HC-toxin production and requires several other *TOX2* genes. ToxF is a putative branched-chain amino acid transaminase that possibly aminates a precursor of Aeo (Cheng *et al.*, 1999), while ToxC is a fatty acid synthase that is involved in the production of the decanoic acid backbone of Aeo (Cheng *et al.*, 1999; Ahn *et al.*, 2002). ToxA is an MFS transporter that is likely involved in the export and self-protection of the fungus against HC-toxin (Pitkin *et al.*, 1996). All *TOX2* genes are expressed *in vitro* and are induced during spore germination (Jones & Dunkle, 1995). Induction of transcription likely involves ToxE, as this bZIP transcription factor can bind to a 10-base motif found in promoters of all the *ToxA* genes (Pedley & Walton, 2001). However, regulation of this gene cluster is certainly more complex, as *TOXE* is not required for *HTS1* expression. A gene cluster in *Fusarium semitectum* was characterized for the production of the cyclic tetrapeptide HDAC inhibitor apicidin (Jin *et al.*, 2010). This gene cluster consists of homologs of *HTS1*, *TOXA*, *TOXE* and *TOXF*, for which the corresponding mutants display phenotypes similar to those of *C. carbonum* mutants in terms of toxin production. The apicidin gene cluster also contains a α-subunit fatty acid synthase, while ToxC is a β-subunit fatty acid synthase. Comparison of the *TOX2* locus with the apicidin gene cluster provides new clues for HC-toxin biosynthesis. The *TOX2* locus especially, may contain additional genes required for HC-toxin production, such as cytochrome P450 monooxygenases. Sequencing of the *C. carbonum* genome would be needed to fully understand HC-toxin biosynthesis and the evolution of the *TOX2* locus/apicidin gene cluster that led to the production of two different HDAC inhibitors.

Proteinaceous HSTs produced by *Dothideomycetes*

In addition to SM HSTs, some necrotrophic plant pathogens secrete small toxic peptides that induce host necrosis or chlorosis, and can act as key determinants of fungal virulence or pathogenicity (Oliver & Solomon, 2010). These ribosome-synthesized HSTs act as effectors of necrotrophy (Friesen *et al.*, 2008a, b). Much of what is known today on the mode of action of proteinaceous HSTs and their interaction with host susceptibility genes comes from detailed studies on two important *Dothideomycete* necrotrophic wheat pathogens, *Ptr* (Ciuffetti *et al.*, 2010) and *S. nodorum* (Table 2; Friesen & Faris, 2010).

Ptr is the causal agent of tan spot of wheat. The pathogen is known to produce two well characterized proteinaceous HSTs, namely PtrToxA and PtrToxB, which interact in a highly specific manner with the host plant and are important determinants of pathogenicity on wheat (Ciuffetti *et al.*, 2010). PtrToxA and PtrToxB induce necrotic and chlorotic symptoms, respectively, on leaves of susceptible wheat genotypes. Sensitivity to one of these toxins renders the host susceptible to the pathogen, while transfer of each individual toxin into a nonpathogenic isolate of the fungus can restore pathogenicity.

PtrToxA was the first proteinaceous HST ever described from a filamentous fungus (Ballance *et al.*, 1989). The toxin induces extensive necrotic symptoms on the leaves of susceptible wheat genotypes, in a light-dependent manner. Although the protein was originally described in *Ptr*, several lines of evidence support that the gene was horizontally transferred to this fungus from *S. nodorum*, a necrotrophic pathogen of wheat that is frequently found in mixed infections with *Ptr* (Friesen *et al.*, 2006). Indeed, while *S. nodorum* has been long recognized as one of the major and regularly occurring pathogens in this crop, infections by *Ptr* have only been reported since 1941. A highly homologous genomic region of 11 kb, containing the *ToxA* gene and a transposase is shared between the two fungi. *ToxA* from *Ptr* and *S. nodorum* share 99.7% similarity and a similar exon-intron structure, while the ITS sequences of the two fungi are only 83% similar. This high similarity and the absence of the gene in related species are consistent with an interspecific gene transfer (Friesen *et al.*, 2006). Moreover, population analysis revealed larger heterogeneous distribution and higher allelic variation in the *ToxA* gene from natural populations of *S. nodorum* than from populations of *Ptr*. This suggests a founder effect in *Ptr*, supporting the hypothesis that the direction of transfer was most likely from *S. nodorum* to *Ptr*. Population analysis also revealed the presence of *PtrToxA* in *c.* 80% of a worldwide collection of isolates of the fungus (Friesen *et al.*, 2006). However, not all races of *Ptr* can produce this toxin as, so far, the presence of *PtrToxA* has only been detected in races 1, 2, 7 and 8 (Ciuffetti *et al.*, 2010). While virulence in the fungus is conferred by secretion of PtrToxA, sensitivity of wheat to the toxin is conferred by the presence of the dominant *Tsn1* allele (Anderson *et al.*, 1999). Wheat genotypes that lack *Tsn1* are significantly less susceptible to races of *Ptr* that produce PtrToxA. *Tsn1* has been recently cloned and it is shown to be a member of the NBS-LRR class of resistance genes. The encoded Tsn1 disease resistance-like protein contains a NBS, a LRR and a serine/threonine protein kinase domain, all of which are necessary for sensitivity to PtrToxA (Faris *et al.*, 2010).

Table 2. Biological activities of SM non-HSTs produced by species of *Dothideomycetes*

Toxin	Species	Chemical structure	Mode of action	Plant target
Toxin	Species	Chemical structure	Mode of action	Plant target
Cercosporin	*Cercospora* spp.	Perylenequinone	Light-dependent production of ROS, lipid peroxidation	Membrane lipids
Elsinochromes	*Elsinoë* spp.	Perylenequinone	Light-dependent production of ROS, lipid peroxidation	Membrane lipids
Phleichrome	*Cladosporium phlei*	Perylenequinone	Light-dependent production of ROS, lipid peroxidation	Membrane lipids
Cladochrome	*Cladosporium cladosporioides*	Perylenequinone	Light-dependent production of ROS, lipid peroxidation	Membrane lipids
Calphostin	*Cladosporium cladosporioides*	Perylenequinone	Light-dependent production of ROS, lipid peroxidation	Membrane lipids
Shiraiachrome	*Shiraia bambusicola*	Perylenequinone	Light-dependent production of ROS, lipid peroxidation	Membrane lipids
Rubellin and uredinorubellin	*Ramularia collo-cygni*	Anthrachinone derivatives	Light-dependent production of ROS, fatty acid peroxidation	Membrane lipids
Sirodesmin PL	*Leptosphaeria maculans*	ETP	Production of ROS through redox cycling	Multiple cellular targets
Phomalirazine	*Leptosphaeria maculans*	ETP	Production of ROS through redox cycling	Multiple cellular targets
Ophiobolin	*Cochliobolus heterostrophus*	Sesquiterpene	Inhibition of calmodulin	Calmodulin
Bipolaroxin	*Bipolaris cynodontis*	Sesquiterpene	Unknown	Unknown
Solanapyrone	*Ascochyta rabiei*	Decalin	Nonspecific binding to proteins (likely membrane proteins)	Multiple (membrane) proteins
Papyracillic acid	*Ascochyta agropyrina*	1,6-Dioxaspiro[4,4]nonene	Unknown	Unknown
Phyllostictine	*Phyllosticta cirsii*	Oxazatricycloalkenone	Unknown	Unknown
Phyllostoxin	*Phyllosticta cirsii*	Pentasubstituted bicyclo-octatrienyl acetic acid ester	Unknown	Unknown
1-O-β-D (14-hydroxy-4, 12-eicosadienoyl)-glucoside	*Curvularia andropogonis*		Unknown	Unknown
Cytochalasin	Several *Dothiodeomycetes*	Cytochalasins	Blocking of polymerization and elongation of actin	Actin
Herbarumin	*Phoma herbarum*	Nonelide lactone	Inhibition of calmodulin	Calmodulin
Pinolidoxin	*Ascochyta pinoides*	Nonelide lactone	Inhibitor of PAL	PAL
Putaminoxin	*Phoma putaminum*	Nonelide lactone	Unknown	Unknown
Stagonolide	*Stagonospora cirsii*	Nonelide lactone	Likely inhibition of photosynthesis	Unknown
Sphaeropsidin	*Sphaeropsis sapinea* f. sp. *cupressi*; *Diplodia mutila*	Pimarane diterpene	Unknown	Unknown
Sphaeropsidone and episphaeropsidone	*Sphaeropsis sapinea* f. sp. *cupressi*	Dimedone methyl ethers	Unknown	Unknown

PtrToxA is the product of a single copy gene. The premature protein consists of a 22 amino acid signal peptide, followed by a 4.3 kDa pro-domain (amino acids 23–60) that is required for proper folding of the mature protein (Tuori *et al.*, 2000). Both domains are cleaved prior to secretion of the 13.2 kDa mature ToxA protein. Crystallization of native ToxA showed that it adopts a single-domain structural conformation, with a β-barrel fold that presents a solvent-exposed loop containing an arginyl-glycyl-aspartic acid (RGD) cell attachment motif (Sarma *et al.*, 2005). The RGD motif is embedded within a stretch of 10 amino acids (amino acids 137–146) that shares similarity with the mammalian protein vitronectin, a protein known to interact with integrin receptors present on the plasma membranes through its RGD motif (Manning *et al.*, 2004). Mutational analysis showed that the RGD motif of PtrToxA as well as other solvent-exposed amino acid residues are required for the necrosis-inducing activity of the toxin (Meinhardt *et al.*, 2002). Cytological studies revealed that PtrToxA is rapidly internalized into mesophyll cells of sensitive wheat cultivars, a process that is facilitated by the RGD motif (Manning

et al., 2008). In addition, conversion of RGD motif to RGE completely abolishes PtrToxA internalization, toxin activity and development of symptoms (Meinhardt *et al.*, 2002; Manning *et al.*, 2004). These data support the hypothesis that the RGD motif of PtrToxA interacts with a high-affinity binding site present on wheat mesophyll cells to facilitate toxin internalization and eventually cell death. However, the identity of the receptor(s) at the cell membrane as well as the precise mechanism underlying PtrToxA internalization remains to be discovered. In this respect, transcriptional profiling has shown that treatment of sensitive wheat leaves with PtrToxA results in increased transcript levels of several receptors, including lectin-like receptors (Pandelova *et al.*, 2009). Similar receptors have been previously identified to interact with RGD-containing peptides and proteins (Gouget *et al.*, 2006). Consequently, Tsn1 has been suggested to be the receptor for PtrToxA, albeit the Tsn1 protein contains no obvious transmembrane domains and it is thus most likely located intracellularly. This might also explain why yeast two-hybrid experiments have failed to show a direct interaction between Tsn1 and PtrToxA. Instead, it is possible that Tsn1 monitors the condition of a yet to be identified receptor of PtrToxA and mediates PCD after interaction with PtrToxA (Faris *et al.*, 2010), similar to what has been proposed as the guard hypothesis in biotrophic pathogen-host interactions (Jones & Dangl, 2006). Finally, based on cytological experiments, endocytosis has also been suggested as a mechanism for internalization as treatment with endocytosis inhibitors prevented PtrToxA internalization (Manning *et al.*, 2008).

After internalization, PtrToxA localizes inside the chloroplasts where it interacts with a protein designated as ToxA binding protein 1 (ToxABP1; Manning *et al.*, 2007). The interaction between PtrToxA and ToxABP1 is mediated through the vitronectin-like loop of PtrToxA but does not require the RGD motif present in this loop. Both transcript and protein levels of ToxABP1 are light-regulated, as is PtrToxA-induced PCD (Manning *et al.*, 2007). Database searches revealed that ToxABP1 is conserved in several cyanobacteria and plants, including rice, potato and *A. thaliana*. It is shown that the *ToxABP1* homolog in *A. thaliana*, *Thf1*, is localized in both chloroplasts and stromules (Wang *et al.*, 2004). Stromules are extrusions of plastids connecting them to other membrane systems, including the plasma membrane. Thus, it is possible that after internalization, ToxABP1 provides a route for PtrToxA from the plasma membrane to the chloroplasts, where it likely functions. Functional analysis of *Thf1* and *Psb29*, the *ToxABP1* homolog in the blue-green alga *Synechocystis* (Keren *et al.*, 2005), revealed that knock-out mutants show a reduced growth rate, loss of chlorophyll, reduced photosystem II (PSII) activity with increasing light intensities, impaired chloroplast development, and a lack of organized thylakoids (Wang *et al.*, 2004; Keren *et al.*, 2005). Interestingly, treatment of sensitive wheat cells with PtrToxA also leads to the disruption of the photosynthetic machinery in a light-dependent manner, loss of chlorophyll and the disorganization of thylakoids (Manning *et al.*, 2007). These similarities suggest that the PtrToxA mode of action is associated with disruption of the photosynthetic machinery by interfering with chloroplast function. Thus, it seems that PtrToxA induces alterations in photosystems I and II (PSI and PSII) by interacting with ToxABP1, leading to light-dependent accumulation of ROS in chloroplasts that disrupts their photosynthetic capacity and triggers PCD (Manning *et al.*, 2009; Pandelova *et al.*, 2009). This hypothesis is supported by the light-dependence of PCD activity of PtrToxA and the light-regulated transcription of *ToxABP1* and *Tsn1*. Moreover, inhibition of ROS accumulation by the antioxidant *N*-acetyl cysteine abolishes the onset of PCD, suggesting that the observed light-depended accumulation of ROS in the chloroplasts is required for PtrToxA-induced PCD (Manning *et al.*, 2009). However, silencing of *ToxABP1* does not completely abolish the development of PtrToxA-induced PCD and it is likely that additional plant proteins interact with PtrToxA or are necessary for full toxin activity (Ciuffetti *et al.*, 2010).

PtrToxB is the second proteinaceous HST from *Ptr* also known as chlorosis-inducing toxin. The toxin is predominantly produced by race 5 isolates of the fungus (Lamari *et al.*, 2003). Unlike the single-copy *PtrToxA*, *PtrToxB* is present in multiple copies, ranging from two to up to ten in different races of the fungus (Martinez *et al.*, 2004; Strelkov *et al.*, 2006). Higher *PtrToxB*-copy number causes higher transcript and toxin production levels, which relates to increased symptom development and virulence on susceptible wheat plants (Amaike *et al.*, 2008). For instance, high virulence of race 5 *Ptr* isolates positively correlates with high levels of *PtrToxB* transcripts produced by eight to ten copies of *PtrToxB* present in these isolates, whereas weakly pathogenic isolates of race 5 possess only two copies of *PtrToxB* (Strelkov *et al.*, 2006). High transcript levels of *PtrToxB* also result in higher numbers and more rapid development of appressoria, suggesting that apart from its capacity to induce chlorosis in toxin-sensitive wheat, PtrToxB may also play a role in the basic pathogenicity of the fungus (Aboukhaddour *et al.*, 2012). Interestingly, races 3 and 4 of the fungus that are nonpathogenic on PtrToxB-sensitive wheat genotypes appear to possess a single-copy of a non-functional homolog of *PtrToxB*, termed *toxb*. Although *toxb* is transcriptionally active, its expression levels are considerably lower as compared to wild-type isolates and the produced peptide is not biologically active and does

not induce chlorosis in PtrToxB-sensitive wheat (Martinez *et al.*, 2004; Amaike *et al.*, 2008).

PtrToxB encodes a small secreted pre-protein of 87 amino acids, consisting of a 23 amino acid signal peptide that is cleaved to yield a mature peptide of 64 amino acids with a predicted molecular mass of 6.5 kDa. Four cysteine residues are present in the mature PtrToxB, which are predicted to form two disulfide-bridges and likely generate a tightly folded tertiary structure, rendering the peptide resistant to heat, organic solvents and proteases (Ciuffetti *et al.*, 2010). Such characteristics are frequently found in secreted fungal apoplastic effectors (Stergiopoulos & de Wit, 2009), and it is hypothesized that PtrToxB may also be active in the apoplast (Ciuffetti *et al.*, 2010). Apart from the four cysteine residues, there are no other structural features present in PtrToxB that could point to a particular mode of action. Domain-swaps between the N-terminal, C-terminal and central parts of the wild-type PtrToxB and the inactive toxb protein from race 4 were performed to determine the active domains involved in the induction of chlorosis (Betts *et al.*, 2011). The two homologs share *c.* 86% and 81% similarity and identity, respectively, with most of the variation present in the mature part of the two proteins. The analysis showed that, although all of the PtrToxB protein domains are required for full biological activity, the N-terminal region has the greatest impact on PtrToxB chlorosis-inducing activity (Betts *et al.*, 2011). Site-directed mutagenesis of the two amino acids in the N-terminal region that differ between PtrToxB and toxb showed both of them to be essential for full PtrToxB activity and the sequence surrounding the first cysteine residue necessary for proper folding of the protein (Betts *et al.*, 2011). Thus, PtrToxB does not contain a single contiguous motif required to trigger chlorosis in sensitive wheat genotypes. PtrToxB-induced chlorosis is light-dependent and likely involves the production of ROS, in this way resembling the mode of action of PtrToxA (Strelkov *et al.*, 1998). Similar to PtrToxA, it is proposed that PtrToxB causes a rapid disruption of the photosynthetic processes in sensitive wheat, leading to the generation of ROS and oxidative stress, chlorophyll photo-oxidation and the development of chlorosis (Kim *et al.*, 2010). The early chlorosis developed by PtrToxB coincides with induced degradation of chlorophyll (Strelkov *et al.*, 1998). Gene expression profiling and biochemical studies also support the role of ROS production and chloroplast involvement in PtrToxB-triggered chlorosis (Ciuffetti *et al.*, 2010). Additionally, comparative microarray analysis between PtrToxA- and PtrToxB-induced responses illustrated that the two different toxins evoke similar host defense responses (Ciuffetti *et al.*, 2010), suggesting that the cellular targets of PtrToxA and PtrToxB and their mode of actions are fairly similar.

Unlike other proteinaceous HSTs, PtrToxB homologs are present across a broad range of plant pathogenic *Ascomycete* fungi, including species of *Cochliobolus*, *Alternaria*, and sister-species of *Pyrenophora* like *P. bromi* (Andrie *et al.*, 2008). Surprisingly, the taxonomic distribution of PtrToxB extends even outside the order of *Pleosporales*, to fungi such as *Magnaporthe grisea*, a member of the *Sordariomycetes*. This suggests that PtrToxB has been inherited from a common ancestral species that predates the split between *Dothideomycetes* and *Sordariomycetes*. *Pyrenophora bromi* is the causal agent of brown leaf spot of smooth bromegrass (*Bromus inermis*) and as in *Ptr*, different isolates of the fungus possess multiple copies of ToxB (Andrie *et al.*, 2008). However, in contrast to PtrToxB, the ToxB alleles within and between isolates of *P. bromi* are not necessarily identical. Furthermore, *P. bromi* ToxB isoforms do not induce chlorosis on the pathogen's host smooth bromegrass but they are able to develop chlorosis in ToxB-sensitive wheat cultivars (Andrie *et al.*, 2008).

The necrotrophic pathogen *S. nodorum* (anamorph *Phaeosphaeria nodorum*) causes *S. nodorum* glume blotch (SNB) on wheat, one of the major and economically important diseases in wheat growing areas of the world. To date, five HSTs have been identified from *S. nodorum,* including SnToxA, SnTox1, SnTox2, SnTox3 and SnTox4. The corresponding host susceptibility genes have been identified as *Tsn1*, *Snn1*, *Snn2*, *Snn3* and *Snn4*, respectively (Friesen *et al.*, 2008a, b). Both additive and epistatic effects have been reported when multiple compatible HST sensitivity gene interactions segregate in the same population (Friesen *et al.*, 2008a, b). In this respect, the ToxA-*Tsn1*, SnTox1-*Snn1*, SnTox2-*Snn2*, SnTox3-*Snn3* and SnTox4-*Snn4* interactions were shown to account for as much as 95%, 58%, 47%, 17% and 41% of the variation in SNB development, respectively (Friesen *et al.*, 2007, 2008a, b). Thus, each of the five HSTs characterized so far is contributing to the development of SNB. The *SnToxA* gene will not be discussed here as it is nearly identical to *PtrToxA*. As discussed previously the latter has been gained by *Ptr,* by horizontal gene transfer from *S. nodorum* (Friesen *et al.*, 2006) and overall, SnToxA and PtrToxA have comparable modes of action.

SnTox1 was the first HST to be described from *S. nodorum* (Liu *et al.*, 2004). The toxin induces necrosis on wheat genotypes carrying *Snn1*, a single dominant gene (Liu *et al.*, 2004). *Snn1* has not been cloned yet, although high-resolution mapping identified two EST-based markers as candidate genes that co-segregate with *Snn1* and encode an NBS-LRR type of resistance protein (Reddy *et al.*, 2008). *SnTox1* encodes a protein of 117 amino acids, consisting of a predicted 17 amino acid

signal peptide and a 100 amino acid cysteine-rich mature peptide with an estimated molecular mass of 10 kDa (Liu *et al.*, 2012). Sixteen cysteine residues are present in the mature protein, a feature commonly found in apoplastic fungal effectors, suggesting that SnTox1 may also function in the apoplast (Liu *et al.*, 2012). Interestingly, SnTox1 possesses a chitin-binding domain (ChtBD) with a conserved C-terminal chitin-binding motif that is mostly present in plant chitin-binding proteins (ChtBD1 or CBM18 superfamily). This domain is different from the ChtBD present in the apoplastic Avr4 effector protein (ChtBD2 or CBM14 superfamily) from the tomato pathogen *Cladosporium fulvum* (Stergiopoulos & de Wit, 2009). It is unknown whether the ChtBD1 present in SnTox1 is functional in binding chitin (Liu *et al.*, 2012). A global population analysis involving 777 isolates of *S. nodorum* showed that *SnTox1* is the predominant toxin gene present in these isolates (*c.* 85%) as compared to other *S. nodorum* toxin genes, such as *SnToxA* (*c.* 36%) and *SnTox3* (*c.* 60%). However, *SnTox1* was absent from all *S. nodorum* isolates that were collected from wild grasses and other taxonomically related species such as *Ptr*, *P. teres*, *P. bromi* and *M. graminicola* that were nonpathogenic on wheat (Liu *et al.*, 2012). Analysis of sequence variation in *SnTox1* identified at least 12 different nucleotide haplotypes, 11 of which encode different protein isoforms indicating that SnTox1 is under diversifying selection. However, none of the characterized nonsynonymous substitutions were present in the putative ChtBD as was reported for *C. fulvum* Avr4 (Stergiopoulos *et al.*, 2007). Deletion of *SnTox1* from virulent isolates of the fungus renders them nonpathogenic on *Snn1* wheat lines while conversely, expression of the *SnTox1* gene in nonpathogenic isolates renders them pathogenic on *Snn1* wheat lines (Liu *et al.*, 2012). Studies on the mode of action of SnTox1 suggest that it is similar to SnToxA. Indeed, *SnTox1*, *SnToxA,* as well as *SnTox3*, all have similar expression patterns with transcript abundance peaking at 3 days post inoculation (dpi), which correlates with the onset of necrosis development (Liu *et al.*, 2009, 2012; Faris *et al.*, 2011). SnTox1-induced PCD and disease susceptibility in *Snn1* lines are also light-dependent as is the case for the SnToxA-*Tsn1* interaction (Friesen *et al.*, 2007). Comparative analysis of the infection process between nonpathogenic and pathogenic isolates of *S. nodorum* has shown that SnTox1 plays a significant role during the initial penetration process. Most likely, SnTox1 is required to trigger *Snn1*-mediated PCD of epidermal cells thus providing the fungus with nutrients for further invasive growth (Liu *et al.*, 2012).

SnTox2 is the third proteinaceous HST identified from *S. nodorum*. The toxin causes necrosis on wheat genotypes carrying the *Snn2* sensitivity gene (Friesen *et al.*, 2007). SnTox2 has only partially been characterized and its molecular mass has been estimated to be between 7 and 10 kDa. The toxin is protease-sensitive and similar to SnToxA and SnTox1 its ability to trigger PCD in susceptible wheat genotypes is light-dependent (Friesen *et al.*, 2007). This suggests that all three toxins may recruit similar host components to induce PCD. A wheat recombinant inbred population segregating for *Snn2* and *Tsn1* (ToxA sensitivity) showed that the SnTox2–*Snn2* interaction accounts for 47%, while the SnToxA–*Tsn1* interaction accounts for 20% of the disease variation (Friesen & Faris, 2010). The two interactions were almost completely additive and together accounted for 65% of the disease variation. The additive effects conferred by multiple interactions of toxins with cognate sensitivity genes might explain why necrotrophic pathogens like *S. nodorum* produce so many different toxins (Friesen *et al.*, 2007; Friesen & Faris, 2010).

SnTox3 induces PCD on wheat genotypes carrying the *Snn3* toxin-sensitivity locus. *SnTox3* is a single copy, intronless gene encoding a pre-protein of 230 amino acids with an estimated molecular mass of 26 kDa (Liu *et al.*, 2009). After removal of a 20 amino acid signal peptide and a predicted pro-sequence, the mature SnTox3 contains six cysteine residues that most likely are involved in the formation of three disulfide bonds, as treatment of *Pichia pastoris*-produced SnTox3 with the reducing agent dithiothreitol (DTT) destroyed PCD activity. No homologs of *SnTox3* have been found yet in other microbes, while the distribution of *SnTox3* in natural populations of *S. nodorum* shows variable deletion frequencies in different populations from around the world and with a global presence frequency of *c.* 60.1%. In total, 11 nucleotide substitutions have been identified in *SnTox3* from global populations of the fungus, which however, give rise to only four haplotypes of the produced protein (Liu *et al.*, 2009). Expression profiling of *SnTox3* showed that similar to *SnToxA*, transcription of this gene peaks at 3 dpi and decreases dramatically from 5 dpi onwards. This time frame coincides with hyphal proliferation at 3 dpi and the onset of symptom development, and at 5 dpi with transition from hyphal growth to asexual sporulation (Liu *et al.*, 2009; Friesen & Faris, 2010). *SnTox3*-disruption caused decrease in virulence of the fungus on *Snn3* wheat lines, whereas heterologous expression of *SnTox3* in a nonpathogenic strain of *S. nodorum* was sufficient to render this strain virulent on *Snn3* lines (Liu *et al.*, 2009; Friesen & Faris, 2010). Together these results demonstrate that SnTox3 is an important virulence factor for the fungus on wheat lines carrying the *Snn3* gene. Almost nothing is known about the mode of action of SnTox3 but the nature of its interaction with *Snn3* seems to be different from the previously discussed SnToxA–*Tsn1*, SnTox1–*Snn1*, and

SnTox2–*Snn2* interactions. Indeed, SnTox3 activity on *Snn3* wheat is not light-dependent and the toxin is less important for disease development, as it accounts for a maximum of only 17% of the disease variation (Friesen *et al.*, 2008a, b; Friesen & Faris, 2010). The relatively low impact of the SnTox3–*Snn3* interaction can easily be masked by other key interactions, like the SnTox2–*Snn2* and SnToxA–*Tsn1* interactions. In fact, in the presence of the SnToxA–*Tsn1* interaction the SnTox3–*Snn3* interaction is practically undetectable (Friesen *et al.*, 2008a, b; Friesen & Faris, 2010).

SnTox4 is the most recently identified proteinaceous HST from *S. nodorum*. The toxin has only partially been characterized and its molecular mass is estimated between 10 and 30 kDa (Abeysekara *et al.*, 2009). Sensitivity of wheat to SnTox4 is governed by *Snn4*, a single dominant gene (Abeysekara *et al.*, 2009). Just like the previously described interactions between ToxA–*Tsn1*, SnTox1–*Snn1*, and SnTox2–*Snn2*, the SnTox4–*Snn4* interaction is also light-dependent. However, this interaction results in the development of only mottled necrotic symptoms as compared to the severe and extensive necrosis caused by the other proteinaceous *S. nodorum* HSTs. The *Snn4* locus is responsible for 41% of the phenotypic variation associated with susceptibility to SNB indicating that the SnTox4–*Snn4* interaction is a significant factor in the development of the disease.

Non-HSTs produced by plant pathogenic *Dothideomycetes*

Dothideomycetes are well known for the production of HSTs, but they also produce many non-host-specific and highly toxic SMs (Table 2; Fig. 2). Perylenequinones, are major a class of photo-activated polyketide non-HSTs that include the well-studied cercosporin and elsinochrome toxins. These dark red pigments contain a highly oxidized pentacyclic core and harbor an axial chirality owing to their helical structure (Mulrooney *et al.*, 2010). Cercosporin, produced by species of *Cercospora* was the first perylenequinone (Fig. 2) isolated from fungi (Daub, 1982). Since then, more than 20 perylenequinones have been characterized from various fungi, most of them belonging to the *Dothideomycetes* (Daub *et al.*, 2005). In

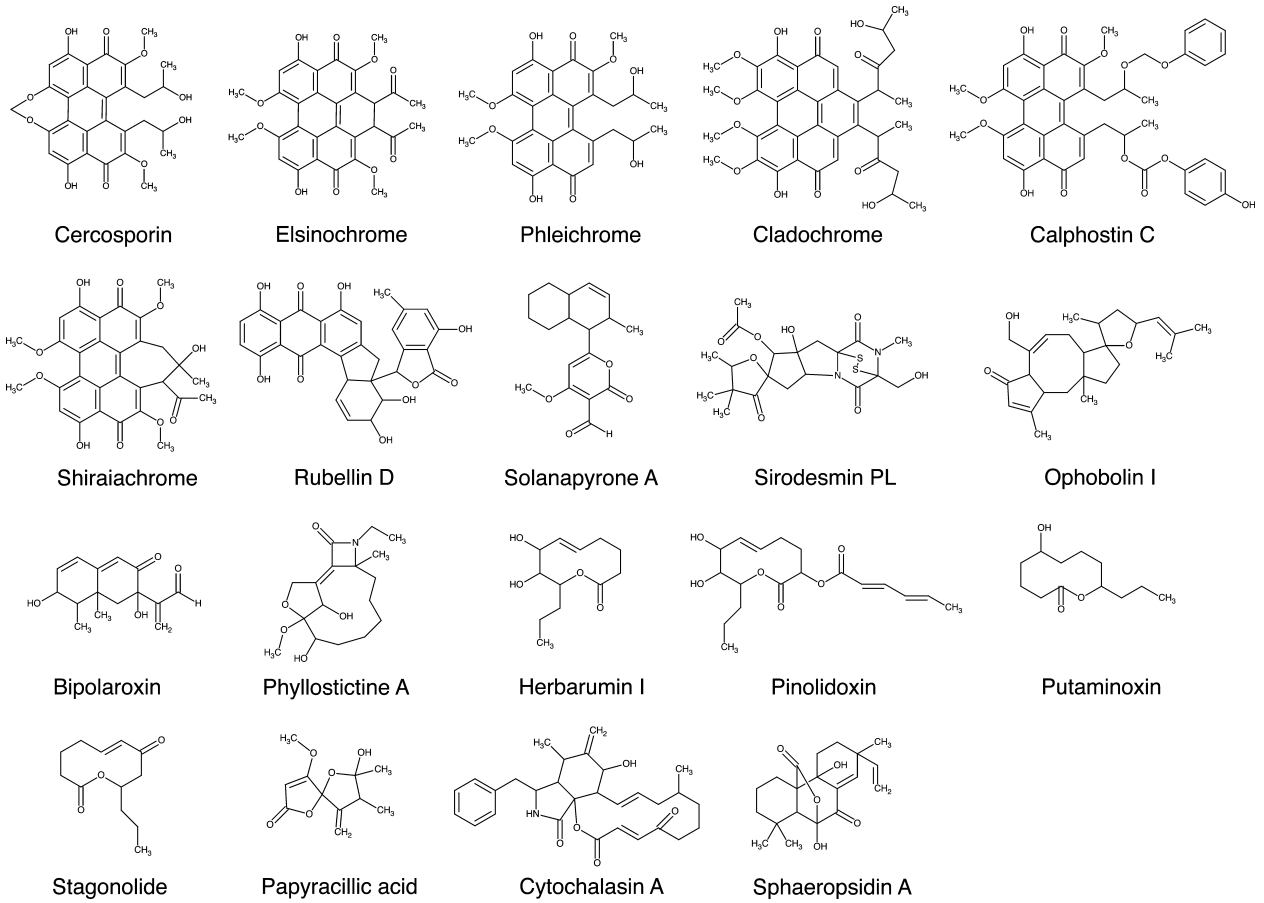

Fig. 2. Structures of SM non-HSTs produced by fungal species of *Dothideomycetes*.

addition to being phytotoxic, some of these compounds have other interesting properties as well, including inhibition of protein kinase C (calphostins; Barak *et al.*, 1991), antiviral activity (calphostin C; Hudson *et al.*, 1997) and anti-proliferative effects on tumor cell lines (cercosporin and calphostin C; Vandenbogaerde *et al.*, 1998). Owing to their photo-activation, perylenequinones are potent photosensitizing agents that could be used in photodynamic therapy (Olivo & Chin, 2006).

Cercosporin was first isolated from the fungus *Cercospora kikuchii*, the causal agent of the 'purple speck' disease of soybean. Since then, the toxin has been isolated from many *Cercospora* species growing *in vitro* or *in planta*, indicating that plant pathogenic strains produce this compound during infection of the host. Cercosporin is phytotoxic on diverse plant species, including *Nicotiana tabacum* and *Phaseolus vulgaris*, implying that this is a non-HST (Fajola, 1978). In addition, cercosporin has antimicrobial properties against bacteria (Fajola, 1978). An important role of cercosporin in pathogenicity was demonstrated for *C. kikuchii* as mutants of the fungus impaired in cerscosporin production could not trigger lesions on soybean (Upchurch *et al.*, 1991). Similarly, in *Cercospora nicotianae*, cercosporin deficient knock-out mutants trigger fewer necrotic lesions on tobacco as compared to the wild type (Choquer *et al.*, 2005).

Elsinochromes (A, B, C and D) are perylenequinone (Fig. 2) pigments synthesized by many *Elsinoë* species, including *Elsinoë fawcettii* and *Elsinoë australis* that are important pathogens of various citrus species (Chung, 2011). Elsinochrome is toxic to citrus and tobacco cell suspensions (Liao & Chung, 2008a, b) and can be isolated from necrotic lesions of infected citrus leaves (Wang *et al.*, 2009), suggesting an important role for pathogenicity. Although no correlation could be found between *in vitro* production levels of elsinochrome and pathogenicity on the host (Wang *et al.*, 2009), this toxin is required for full virulence. Indeed, *E. fawcettii* knock-out mutants unable to produce elsinochrome trigger fewer necrotic lesions as compared to the wild type (Chung & Liao, 2008; Liao & Chung, 2008a, b). Finally, the bindweed bio-control fungus *Stagonospora convolvuli* are also reported to produce elsinochrome (Chung, 2011).

Several *Dothideomycetes* are known to produce photoactive compounds that are similar to cercosporin and elsinochrome, including phleichrome, calphostin, cladochrome and shiraiachrome (Arnone *et al.*, 1988; Mulrooney *et al.*, 2010). *Cladosporium phlei* is the causal agent of the purple spot disease of timothy and a producer of phleichrome, a natural product with both phytotoxic and antimicrobial properties (Fig. 2; Araki & Shimanuki, 1983). While the role of phleichrome in pathogenesis is not yet proven, it is shown that phleichrome production is induced by diketopiperadine compounds produced by *Epichloe typhina*, an endophytic fungus also found on timothy (Seto *et al.*, 2005). Phleichrome is toxic to *E. typhina* in the presence of light, suggesting a role for this toxin in antagonistic interactions between the two fungi. The mild pathogen of wheat *Cladosporium cladosporioides* is known for its *in vitro* production of the perylenequinone compounds cladochromes and calphostins (Fig. 2; Williams *et al.*, 2008). Previously, only cladochromes A and B were reported from *Cladosporium cucumerinum* during infection of cucumber (Arnone *et al.*, 1988). These, and other cladochrome derivatives, can be isolated from *in vitro* growing cultures of the fungus only with the combined use of chemical inducers that modify chromatin conformation, suggesting that they play a role during pathogenesis. The chemical synthesis of calphostins and their cytotoxicity on animal cells have been extensively studied owing to their protein kinase C inhibitory and antitumor activities (Barak *et al.*, 1991; Mulrooney *et al.*, 2010). Finally, shiraiachrome A and B are produced by the bamboo pathogen *Shiraia bambusicola* (Fig. 2; Wang *et al.*, 1992). Like other perylenequinones derivatives, these compounds show antitumor, antiviral and protein kinase C inhibitory activities (Wang *et al.*, 1992). Shiraiachrome A also exhibits anti-angiogenic properties, which explains the use of *S. bambusicola* in the treatment of rheumatoid arthritis in Chinese folk medicine (Tong *et al.*, 2004).

The mode of action of perylenequinones has been extensively studied, mainly owing to their light activated properties that could be exploited as photosensitizers in photodynamic therapy. All fungal perylenequinones share a basic 3,10-dihydroxy-4,9-perylenequinone chromophore that can absorb light-energy (Daub *et al.*, 2005). This results in conversion of the compound to an electron-active triplet state that can be subsequently reduced to react with cellular molecules or oxygen to generate singlet oxygen (e.g. production of ROS, such as superoxide anion, hydrogen peroxide and hydroxyl radicals; Daub *et al.*, 2005). The mode of action and cell damage invoked by perylenequinones have been extensively studied using mainly cercosporin as a model. This compound is not specifically toxic to any particular cell type, as it relies on the generation of toxic ROS products that are harmful to bacterial, fungal, plant and animal cells. The studies have shown that it induces lipid peroxidation of liposomes, microsomes, rat liver, membrane lipids of tobacco cells and pea internode mitochondria (Cavallini *et al.*, 1979; Daub, 1982). In particular, the toxin destroys unsaturated acyl chains of lipids that consequently trigger extensive structural changes in membranes (Daub & Briggs, 1983). Lipid peroxidation occurs rapidly after application of the toxin, with electrolyte leakage observed within

2 min and cell damage within 45 min upon exposure to light. These effects however, can be inhibited by singlet oxygen quenchers, suggesting that lipid peroxidation involves the production of singlet oxygen (Cavallini *et al.*, 1979; Daub & Briggs, 1983). Similarly to cercosporin, elsinochrome induces electrolyte leakage in a light-dependent manner (Liao & Chung, 2008a, b). In this case, the use of singlet oxygen quenchers confirmed the production of singlet oxygens upon exposure to light (Liao & Chung, 2008a, b). Finally, both cercosporin and elsinochrome are known to induce the production of superoxide in a light-dependent manner (Daub & Briggs, 1983; Liao & Chung, 2008a, b).

The basis of cell resistance to perylenequinones was studied using cercosporin as a model, and diverse mechanisms have been identified that play a role in resistance against this compound and consequently against *Cercospora* species as well. One mechanism seems to be reductive detoxification of cercosporin by cercosporin-resistant fungi (Daub *et al.*, 2005). Indeed, these fungi show a higher reduction activity of diverse dyes with different redox potentials than cercosporin-sensitive fungi, such as *Aspergillus flavus* and *Neurospora crassa* (Daub *et al.*, 1992). Accordingly, over-expression of the flavin adenine dinucleotide (FAD)-dependent pyridine nucleotide reductase Cpd1p in *Saccharomyces cerevisiae* resulted in resistance to cercosporin (Ververidis *et al.*, 2001). This mechanism of detoxification that partially contributes to resistance to singlet oxygen-generating compounds is active locally around the hyphae and reversible, allowing the toxin to spontaneously re-oxidize into a form that is toxic for the plant cell (Daub *et al.*, 2005). Transgenic tobacco plants expressing *CDP1* are resistant to cercosporin, indicating that this gene could be used as a source of resistance against cercosporin-producing pathogens (Panagiotis *et al.*, 2007). Also, several bacteria species have the ability to degrade cercosporin by removing one methoxyl group and adding one oxygen atom that generates an unstable green metabolite (xanosporic acid) with no activity against cercosporin-sensitive fungi and plant cells (Mitchell *et al.*, 2002). Mutant screening and expression profiling in the presence of cercosporin suggested that an oxidoreductase is involved in cercosporin degradation by the bacterial species *Xanthomonas campestris* pv. *zinniae* (Taylor *et al.*, 2006). However, expression of this oxidoreductase in noncercosporin-degrading bacteria did not result in cercosporin degradation, suggesting that other factors are required as well. Identification of the complete set of bacterial genes that degrade cercosporin is of interest for the genetic engineering of plants resistant to cercosporin-producing pathogens.

An additional mechanism of resistance to cercosporin that is commonly found in fungi is based on active efflux of the toxin. At least three membrane transporters, namely cercosporin facilitator protein (CFP), Ctb4 and Atr1 are implicated in transport of the toxin in various *Cercospora* species (Callahan *et al.*, 1999; Choquer *et al.*, 2007; Amnuaykanjanasin & Daub, 2009). The MFS transporter CFP is involved in secretion and self-protection against cercosporin in *C. kikuchii* (Callahan *et al.*, 1999; Upchurch *et al.*, 2001) and likely in *C. nicotianae* as well (Amnuaykanjanasin & Daub, 2009). Ctb4 is also an MFS transporter that is encoded by a gene located within the cercosporin biosynthesis gene cluster (Chen *et al.*, 2007a, b). However, although secretion of cercosporin and consequently virulence are impaired in *Δctb4* deletion mutants, these mutants are still tolerant to the toxin (Choquer *et al.*, 2007). Finally, Atr1 is an ABC transporter involved in cercosporin export and partial self-protection against it, as disruption and overexpression mutants of the fungus are slightly more sensitive or tolerant to the toxin, respectively (Amnuaykanjanasin & Daub, 2009). Further analyses of these three transporters are needed to better understand their roles in cercosporin export and resistance. *CRG1* is a Zn(2)Cys(6) transcription factor that among others regulates the expression CFP, which explains its role in resistance against cercosporin. However, over-expression of CFP in a *Δcrg1* mutant only results in a slight increase in tolerance to cercosporin (Amnuaykanjanasin & Daub, 2009), suggesting that several transporters, and other mechanisms as well, are involved in resistance to cercosporin.

A third mechanism used by *C. nicotianae* for self-protection against cercosporin is based on pyridoxine production (vitamin B6), a quencher of singlet oxygen. Pyridoxine biosynthesis is controlled by the *SOR1* gene (Ehrenshaft *et al.*, 1998), which is found in various perylenequinone producing fungi (Ehrenshaft *et al.*, 1999). Pyridoxine quenches singlet oxygen at similar rates to other efficient antioxidants, as for example vitamins C and E (Ehrenshaft *et al.*, 1999). In addition, pyridoxine is able to quench superoxide (Jain & Lim, 2001).

Plants also developed antioxidant defense mechanisms, including catalases and superoxide dismutases (SODs). Transcriptome analysis in maize after treatment with cercosporin showed significant increase in *SOD* gene expression (Williamson & Scandalios, 1992). These genes are certainly good candidates for engineering of plants with resistance to cercosporin. Indeed, transgenic sugar beets expressing *SOD* genes from tomato show increased resistance to cercosporin, and consequently to infection by *Cercospora beticola* (Tertivanidis *et al.*, 2004).

Rubellins and uredinorubellins are also photoactivated compounds produced by the plant pathogenic fungi *Ramularia collo-cygni* and the hyper-parasite *Ramularia uredinicola*, respectively (Miethbauer *et al.*, 2008). Although these molecules have a different structure as compared to

perylenequinones (Fig. 2), they show a similar type of photo-activation, suggesting a similar mode of action. However, a role in virulence for both compounds is not yet known.

Another class of non-HSTs are the epipolythiodioxopiperazine (ETP) compounds. These SMs are characterized by a bridged disulfide piperazinedione six-membered ring, derived from a cyclic dipeptide, which seems to be required for their biological activity (Mullbacher *et al.*, 1986). Their potential use as chemotherapeutic agents provoked investigations on their mode of action, and studies were mainly performed on gliotoxin, a toxin produced by the human opportunistic pathogen *Aspergillus fumigatus*. Nevertheless, a few ETP toxins have been isolated from *Dothideomycete* fungi as well. In this respect, sirodesmin PL produced by *L. maculans* is one of the most well-studied ETP phytotoxins (Fig. 2; Rouxel *et al.*, 1988). Phytoxicity of sirodesmin PL suggests a role for this toxin in blackleg disease on *Brassica napus* (Rouxel *et al.*, 1988). Knock-out mutants unable to produce sirodesmin do not completely abolish pathogenicity but show decreased virulence (Gardiner *et al.*, 2004; Elliott *et al.*, 2007). In addition to phytotoxicity, sirodesmin PL also shows antimicrobial activity toward gram-positive bacteria, which relies on the disulfide bridge structure of the toxin (Boudart, 1989). An analog of sirodesmin PL that likely originates from the same biosynthetic pathway is phomalirazine (Pedras *et al.*, 1989), produced by the fungus *P. lingam*, showing that several bioactive compounds can originate from a single pathway.

The mode of action of ETPs like sirodesmin PL has been mainly studied using gliotoxin as a model. Toxicity of ETPs likely involves the sulfur atoms in the central diketothiopiperazine moiety that either inactivate proteins by conjugation with thiol residues or generate ROS (Gardiner *et al.*, 2005). Several examples of covalent interaction with proteins have been reported for gliotoxin, but so far, none for the ETPs produced by plant pathogenic fungi. These toxins do not exhibit target specificity and thus can disrupt many different functions in the cell. For example, gliotoxin and other ETPs can act as immunosuppressors as the transcription factor NF-κB that is involved in immune responses is a target of these compounds (Pahl *et al.*, 1996). The immunosuppressor activity may also be owing to inhibition of the chymotrypsin-like activity of the 20S proteasome (Kroll *et al.*, 1999). ETPs can also trigger PCD (Waring *et al.*, 1988), which appears dependent on caspase-3-like activity and production of ROS (Zhou *et al.*, 2000). Mitochondrial proteins are also targeted by ETPs, as shown by the decreased rate of ATP synthesis owing to inhibition of ATP-synthases, and the induced alterations in their function is coupled to the apoptosis triggered by gliotoxin (Gardiner *et al.*, 2005). However, induction of ROS by gliotoxin and other ETPs is not linked with apoptosis because quencher molecules do not inhibit PCD (Waring *et al.*, 1995). Overall, stimulation of ROS appears to be of little importance to explain the PCD activity of ETPs, especially because the low concentration of gliotoxin required to induce apoptosis is not sufficient to induce an oxidative stress (Gardiner *et al.*, 2005). Instead, PCD activity of ETPs could be explained by their effect on calcium homeostasis as gliotoxin interacts with thiol residues of a redox sensitive plasma membrane calcium channel (Hurne *et al.*, 2002). This interaction in thymocytes results in an increase in calcium influx that can cause oxidative damage and subsequent necrosis. However, the role of all these processes in toxicity remains uncertain.

Many other toxic compounds are produced by *Dothideomycete* fungi, but their role in pathogenicity has not been proven or has not been studied in great detail. In addition to T-toxin for example, *C. heterostrophus* also produces ophiobolins that are sesquiterpene phytotoxins (Fig. 2) with potential herbicidal and antinematocidal properties (Tsipouras *et al.*, 1996; Evidente *et al.*, 2006). A mechanism for their antimicrobial and antitumor activity was proposed based on competition for binding to the membrane ivermectin receptor, which modulates an invertebrate specific glutamate-gated chloride channel (Tsipouras *et al.*, 1996). Ophiobolins are also active against certain *Zygomycetes* as they inhibit sporangiospore germination of *Mucor circinelloides* and in addition cause morphological defects (Krizsan *et al.*, 2010). The Bermuda grass pathogen *Bipolaris cynodontis* produces two phytotoxic sesquiterpenes named bipolaroxins (Fig. 2). The fatty acid ester of bipolaroxin is an inhibitor of the steroidal plant growth regulator brassinolide, but it is not yet known whether this activity is responsible for toxicity (Kim *et al.*, 1998). Furthermore, the chickpea pathogen *Ascochyta rabiei* is known to produce the phytotoxic polyketides, solanapyrones (Fig. 2), which are suspected to play a role in microbial pathogenesis (Kasahara *et al.*, 2010). Papyracillic acid (Fig. 2) produced by *Ascochyta agropyrina* is toxic to plants, including its host quack grass, which implies that it could be used as a control agent against this noxious perennial weed (Evidente *et al.*, 2009). *Phyllosticta cirsii* is a pathogen of the noxious weed *Cirsium arvense* that produces four oxazatricycloalkenones, collectively known as phyllostictines. These are highly toxic on the host plant and show antimicrobial activity against Gram-positive bacteria as well (Evidente *et al.*, 2008). In addition, *P. cirsii* produces phyllostoxin, a penta substituted bicyclo-octatrienyl acetic acid ester (Fig. 2) that is also highly toxic on its host plant (Evidente *et al.*, 2008). *Curvularia andropogonis* is a pathogen of *Java citronella* and is known to produce a highly toxic 1-*O*-β-D

(14-hydroxy-4, 12-eicosadienoyl)-glucoside *in vitro* and *in planta* (Alam *et al.*, 1997). The purified toxin induces symptoms of the disease on the host plant but it is also toxic to other plants. Finally, many *Dothiodeomycete* fungi produce cytochalasins (Fig. 2), compounds that inhibit actin and could thus be important for plant infection, as plant cells reorganize actin filaments upon contact with pathogens (Scherlach *et al.*, 2010).

Phoma herbarum is a ubiquitous saprophyte that can also be pathogenic on plants and animals. Its virulence is based on the production of diverse natural products, including the phytohormone gibberellin and the phytotoxic herbarumins (Rivero-Cruz *et al.*, 2003). These SMs are nonelide lactones (Fig. 2) that inhibit germination and growth of *Amaranthus hypochondriacus* (Furstner *et al.*, 2002). The compounds are analogs of the phytotoxins pinolidoxin and putaminoxin, produced by the *Dothideomycetes*, *Ascochyta pinoides* and *Phoma putaminum*, respectively (Evidente *et al.*, 1998a, b). Pinolidoxin and putaminoxin (Fig. 2) are toxic on diverse plant species, but show little or no antifungal activity and animal toxicity. Their phytotoxicity is correlated with the integrity of the nonelide ring, and the presence of hydoxyl groups and a propyl side chain (Evidente *et al.*, 1998a, b). Pinolidoxin is a potent inhibitor of phenylalanine ammonia lyase (PAL) activity, which plays a key role in plant defense responses (Furstner *et al.*, 2002). Stagonolide is also a nonelide compound (Fig. 2) produced by *Stagonospora cirsii*, a pathogen of the weed *C. arvense* (Yuzikhin *et al.*, 2007). This toxin shows selectivity as its host plant is more sensitive than the non-hosts tomato, pepper, and cucumber (Yuzikhin *et al.*, 2007). In addition to causing necrosis on leaves, stagonolide is a strong inhibitor of root growth in seedlings of *Asteraceae* species, including *C. arvense*. All these nonelide compounds are potent herbicidal agents that could be used to control noxious weeds such as *C. arvense*.

Sphaeropsidins are phytotoxic diterpenes (Fig. 2) that are produced by *Sphaeropsis sapinea* f. sp. *cupressi* and *Diplodia mutila*, which cause canker diseases of Italian cypress. Tests on the biological activity of these toxins against several species of cypress as well as non-host species such as tomato, mung bean and oak, indicated that these are non-HSTs, able to cause symptoms both on host and non-host plants (Sparapano *et al.*, 2004). In addition to being phytotoxic, sphaeropsidins are also toxic against diverse bacteria and fungi, including known pathogens of cypress such as *Seiridium cardinale*, *Seiridium cupressi* and *Seiridium unicorpe* (Evidente *et al.*, 2011). The antifungal activity of sphaeropsidins could suggest that these toxins have a role in antagonistic activities against other fungal pathogens during infection of the host and thus could be used as potential biological control agents. Next to sphaeropsidins, *S. sapinea* f. sp. *cupressi* is also known to produce two phytotoxic dimedone methyl ethers, named sphaeropsidone and episphaeropsidone (Evidente *et al.*, 1998a, b). Different species of cypress exhibit variable levels of sensitivity to these two metabolites, although in general sphaeropsidone appears to be less phytotoxic than episphaeropsidone. Both metabolites are also able to induce symptoms on non-host plants such as tomato. Symptoms induced by sphaeropsidone and episphaeropsidone on plants are in general less severe than those induced by sphaeropsidins. Finally, both sphaeropsidone and episphaeropsidone show antimicrobial activity against a range of differed fungi, including the cypress pathogens *S. cardinale* and *S. cupressi* although, in all cases sphaeropsidone appeared to be less active than episphaeropsidone (Evidente *et al.*, 1998a, b).

Biosynthesis and regulation of SM non-HSTs

A gene cluster for the biosynthesis of cercosporin was identified in *C. nicotianae* that consists of eight genes, *CTB1*-to-*CTB8* (Chen *et al.*, 2007a, b). *CTB1* encodes a nonreducing type I PKS that is the key enzyme responsible for cercosporin biosynthesis. *Δctb1* knock-out mutants are unable to produce cercosporin and induce fewer and smaller necrotic spots compared with the wild type (Choquer *et al.*, 2005). *CTB2* encodes an *O*-methyltransferase that is required for the biosynthesis of cercosporin and virulence of the fungus (Chen *et al.*, 2007a, b). However, *Δctb2* deletion mutants appear to accumulate another yellow pigment that could correspond to a yet structurally uncharacterized precursor. *CTB3* encodes an *O*-methyltransferase (N-terminus)/FAD-dependent monooxygenase (C-terminus) that is required for cercosporin production and full virulence and similarly to *Δctb2*, the *Δctb3* deletion mutants also accumulate a yellow pigment that is not phytotoxic (Dekkers *et al.*, 2007). Deletion of *CTB5*, *CTB6* and *CTB7* that encode putative FAD/FMN- or NADPH-dependent oxidoreductases also resulted in accumulation of a yellow pigment and absence of cercosporin production (Chen *et al.*, 2007a, b). Accordingly, the mutants triggered fewer lesions compared with the wild-type. These functional analyses determined genes involved in the production of cercosporin, while a biosynthetic pathway that involves oxidation, hydration and methylation steps has been proposed (Chen *et al.*, 2007a, b). However, none of the precursors accumulated in the different mutants has been characterized yet and experimental proof for the proposed route is still unavailable. *CTB4* encodes an MFS transporter that is involved in the secretion of the toxin (Choquer *et al.*, 2007). Indeed, deletion

mutants are defective in both cercosporin biosynthesis and secretion, and accumulate a brown pigment. The lower amount of cercosporin suggests the existence of a feedback inhibition. Remarkably, *CTB4* is not involved in self-protection because the mutants retained normal tolerance to cercosporin. The last gene found in the gene cluster was *CTB8*, which encodes a Zn(2)Cys(6) transcription factor (Chen *et al.*, 2007a, b). *Δctb8* deletion mutants are not able to produce cercosporin and are less virulent than the wild type, mainly owing to the absence in expression of the other seven genes present in the *CTB* cluster. *CTB8* appears to act downstream of the previously identified regulator *CRG1* (Chung, 2003), as *CTB8* (and obviously other *CTB* genes) is down-regulated in a *crg1* disrupted mutant, while *CRG1* expression is not affected in a *Δctb8* mutant (Chen *et al.*, 2007a, b). The suggested feedback inhibition upon accumulation of cercosporin or its precursors has been confined in *Δctb1*, *Δctb2* and *Δctb3* mutant backgrounds that show down-regulation of genes in the cluster (Chen *et al.*, 2007a, b). However, this regulation appears more complex as transcription of *CTB* genes is not modified in the *Δctb4* mutant (Choquer *et al.*, 2007). Induction of CTB genes and production of cercosporin is affected by many environmental stimuli including light, carbon, nitrogen, pH, salts, ions and media composition (Choquer *et al.*, 2005; Chen *et al.*, 2007a, b; Dekkers *et al.*, 2007; You *et al.*, 2008). Signaling in response to these diverse environmental stimuli likely involves global regulators such as VeA, but also a calcium/calmodulin and a MAP kinase pathway (Chung, 2003; Shim & Dunkle, 2003).

Elsinochrome toxin produced by *Elsinoë* species is also synthesized through a polyketide pathway. In *E. fawcettii*, *EfPKS1* encodes a nonreducing PKS that is involved in the biosynthesis of elsinochrome, conidiation, and virulence on rough lemon (Liao & Chung, 2008a, b). Other genes adjacent to *EfPKS1* likely play a role in elsinochrome biosynthesis. *RDT1* encodes a reductase with strong similarity to the 1,3,8-trihydroxynaphthalene reductase that is involved in melanin biosynthesis (Chung & Liao, 2008). *ECT1* encodes a putative membrane transporter that could be responsible for secretion and self-protection against the toxin. A role in elsinochrome production for *PRF1*, a gene encoding a putative prefoldin protein, is unclear. At this locus, other genes encoding hypothetical proteins (*OXR1*, *EfHP1* to *EfHP4*) could be involved in elsinochrome biosynthesis but no function could be predicted (Chung & Liao, 2008). The last gene found at this locus is *TSF1*, which encodes a putative transcription factor with DNA binding motifs that are characteristic of C_2H_2 and Zn(2)Cys(6) zinc finger transcription factors (Chung & Liao, 2008). Similarly to *Δefpks1* mutants, *Δtsf1* deletion mutants were unable to produce elsinochrome and were altered in conidiation and pathogenicity on rough lemon. Accordingly, *EfPKS1*, *RDT1*, *PRF1* and *EfHP1* are down-regulated in these mutants, while expression of *ECT1* and the other genes at this locus is unaltered. This suggests that only four genes are involved in the elsinochrome biosynthetic pathway. However, the regulation of this gene cluster seems to be more complex. Indeed, expression of all these genes is not completely correlated with elsinochrome production under nitrogen limitation, high pH or high glucose concentration (Chung & Liao, 2008). Like cercosporin, *EfPKS1* and elsinochrome production seems also to be regulated by light (Liao & Chung, 2008a, b). Similarly in *Δefpks1* mutants, *RDT1*, *TSF1*, *PRF1* and *ECT1* expression was nearly abolished, suggesting a feedback inhibition regulatory network (Chung & Liao, 2008). In addition to Tsf1, *EfPKS1* (but not *TSF1*) is directly regulated by the EfSte12 transcription factor (Yang and Chung, 2010). *EfSTE12* expression is not affected in both *Δefpks1* and *Δtsf1* deletion mutants. Although *EfPKS1* is down-regulated in a *Δefste12* deletion mutant, this strain is still able to produce a smaller amount of elsinochrome and is not affected in pathogenicity or conidiation (Yang & Chung, 2010). Overall, it seems that production of elsinochrome or intermediates may be involved in conidium differentiation, but the underlying genetic mechanism is still unclear.

The biosynthesis pathway of sirodemin PL in *L. maculans* is determined by a gene cluster that consists of 18 genes, including *sirP* a two-module NRPS-encoding gene (Gardiner *et al.*, 2004). The proposed first step in the sirodemin biosynthetic pathway is the transfer of a dimethylallyl group to either the dipeptide cyclo-L-tyrosyl-L-serine or free L-tyrosine, which leads to the formation of phomamide (Gardiner *et al.*, 2004). *O*-prenylation of L-tyrosine in the presence of dimethylallyl diphosphate is performed by the prenyltransferase enzyme encoded by *SirD* (Gardiner *et al.*, 2004; Kremer & Li, 2010). Consistent with the predicted biosynthetic pathway, the gene cluster contains genes that encode a thioredoxin reductase (*sirT*), an acetyl transferase (*sirH*), two methyl transferases (*sirM* and *sirN*), three cytochrome P450 monooxygenases (*sirB*, *sirC* and *sirE*) and one oxidoreductase (*sirO*; Gardiner *et al.*, 2004). Several genes encode proteins with no obvious function in sirodesmin biosynthesis. The glutathione S-transferase sirG and dipeptidase sirJ could be involved in self-protection during biosynthesis. Finally, *sirI* encodes an aminocyclopropane-1-carboxylic acid synthase and *sirQ*, *sirR* and *sirS* encode putative progesterone 5-b-reductases (Gardiner *et al.*, 2004). Apart from *sirP* that whose disruption results in loss of sirodesmin production (Gardiner *et al.*, 2004) and reduced pathogenicity (Elliott *et al.*, 2007), all other genes from this

gene cluster await functional analysis. The sirodesmin gene cluster also contains a gene encoding an ABC transporter (*sirA*). Disruption of this gene results in induction of *sirP* expression and increased accumulation of sirodesmin in the medium (Gardiner *et al.*, 2005). However, this mutant is more sensitive to sirodesmin, suggesting that sirA is involved in self-protection against the toxin. *SirZ* encodes a Zn(2)Cys(6) transcription factor that regulates sirodesmin production (Fox *et al.*, 2008). Indeed, *sirZ*-silenced mutants show down-regulation of genes from the gene cluster (*sirD*, *sirP* and *sirH*). Recently, the expression of the NRPS (*sirP*) and transcription factor encoding genes (*sirZ*) was shown to be controlled in response to amino acid availability by cpcA (Elliott *et al.*, 2011). A sirodesmin-related compound is gliotoxin, produced by the human pathogen *A. fumigatus*. Remarkably, the gene clusters for ETP biosynthesis in both fungi share similarities, suggesting a common origin (Gardiner *et al.*, 2004). An ETP-like gene cluster was also identified in *Penicillium lilacinoechinulatum* that share similarities with gene clusters in *L. maculans* and *A. fumigatus* (Fox & Howlett, 2008). However, although these gene clusters share similarities, the transcription factor PlgliZ is not able to complement a gliZ mutant in *A. fumigatus* (Fox & Howlett, 2008) and gliA provides resistance to gliotoxin only and not to sirodesmin (Gardiner *et al.*, 2005).

Biosynthesis of the other fungal toxins described previously likely involves gene clusters. For example, a gene cluster involved in solanapyrone production was identified in *Alternaria solani* (Kasahara *et al.*, 2010). This gene cluster consists of six genes (*SOL1* to *SOL6*) that encode a PKS, an *O*-methyltransferase, a dehydrogenase, a transcription factor, a flavin-dependent oxidase, and a cytochrome P450 monooxygenase, respectively. All these activities are required in the proposed biosynthetic pathway leading to solanapyrone (Kasahara *et al.*, 2010), although functional analysis remains to be done. Expressing *SOL1* in *Aspergillus oryzae* and producing Sol5 in *P. pastoris* experimentally confirmed the activities of the PKS (SOL1) and oxidase (SOL5) enzymes. The PKS was shown to produce desmethylprosolanapyrone, but cannot catalyze any Diels–Alder cyclization. This activity was demonstrated for the purified oxidase that catalyzed the formation of solanapyrone A from prosolanapyrone II (Kasahara *et al.*, 2010). Therefore, *SOL5* encodes a diels-alderase enzyme involved in oxidation and cycloaddition of prosolanapyrone. As *A. rabiei* also produces solanapyrones, it is expected that a similar gene cluster is present in its genome.

Conclusions and future prospects

Fungal plant pathogens are divided into necrotrophs and biotrophs according to their feeding behavior. Biotrophs exhibit an intimate interaction with living host cells for feeding, while necrotrophs kill host cells and feed on the nutrients released from them. These different lifestyles require different molecular weaponry. Biotrophs secrete effector proteins that exploit host cells without killing them (Stergiopoulos & de Wit, 2009), whereas necrotrophs produce SM or proteinaceous HSTs that kill their host plant (Wolpert *et al.*, 2002; Friesen *et al.*, 2008a, b). HSTs induce PCD in specific genotypes of the host that is mediated by dominant HST sensitivity genes. As a necrotrophic pathogen produces a HST to promote disease and the host contains a receptor that is required for HST sensitivity, the relation between necrotrophic pathogens and their host plants is often considered as the inverse of the classical gene-for-gene system, the hallmark of biotrophic plant–pathogen interactions (Stergiopoulos & de Wit, 2009). In a gene-for-gene system, recognition of a biotroph-derived effector protein by a host resistance receptor protein leads to a resistance response known as effector-triggered immunity (ETI) that results in PCD (Jones & Dangl, 2006). Thus, PCD provides resistance to biotrophs but susceptibility to necrotrophs (Fig. 3). It has been suggested that necrotrophic pathogens might have subverted resistance mechanisms acquired by plants to combat biotrophic pathogens (Friesen *et al.*, 2008a, b) and have recruited weaponry to survive the harsh conditions prevailing in dead or dying host tissue. This notion is supported by the fact that resistance genes active against biotrophs on one hand and susceptibility genes active against necrotrophs on the other, both encode similar types of resistance receptor proteins. Plant disease resistance against biotrophs is conferred by genes that encode extra- or intracellular NBS-LRR types of resistance receptor proteins (Maekawa *et al.*, 2011). However, in recent years host genes encoding NBS-LRR proteins with a similar structure as disease resistance proteins have been reported to also mediate sensitivity to SM and proteinaceous HSTs. The *A. thaliana LOV1* gene for example encodes aCC-NBS-LRR protein required for sensitivity to the HST victorin produced by *C. victoriae* (Lorang *et al.*, 2007). Similarly, sensitivity to the HST PC-toxin produced by *P. circinata* is dependent on an NBS-LRR protein (Pc-B)in the host plant sorghum (Nagy & Bennetzen, 2008). Susceptibility of oat to *C. victoriae* producing victorin and resistance to the obligate biotroph *P. coronata* are correlated and it is suggested that the same CC-NBS-LRR protein may mediate PCD leading to susceptibility to *C. victoriae* and resistance to *P. coronata*, respectively. However, this hypothesis still needs additional experimental support. Whereas, LOV1 and Pc-B mediate recognition of a SM HST, Tsn1 mediates recognition of the proteinaceous HST ToxA produced by the necrotrophic pathogens *Ptr* (Sarma *et al.*, 2005) and *S. nodorum* (Friesen *et al.*,

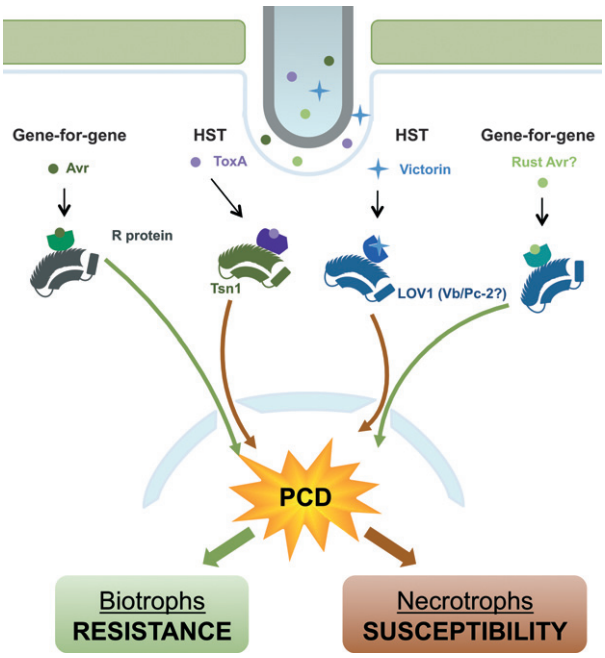

Fig. 3. (CC)-NBS-LRR proteins mediate resistance to (hemi-)biotrophs and susceptibility to necrotrophs. Some plant resistance proteins (R) and proteins that mediate sensitivity to HSTs, both belong to the (CC)-NBS-LRR class of proteins. Avirulence proteins (Avr) secreted by (hemi-)biotrophic pathogens as well as proteinaceous (e.g. ToxA) and secondary metabolite HSTs (e.g. victorin) that are secreted by necrotrophic pathogens, target specific plant proteins. According to the guard model, these targets are guarded by (CC)-NBS-LRR proteins. Binding and concomitant modulation of a host target by an Avr or a HST is sensed by the (CC)-NBS-LRR proteins that mediate PCD, thus providing resistance to a (hemi-)biotroph (green arrows) and susceptibility to a necrotroph (brown arrows). Both cases comply with a gene-for-gene type of interaction. Tsn1 likely senses the binding of the proteinaceous HST ToxA produced by *Pyrenophora tritici-repentis,* to its plant target and mediates PCD resulting in in plant susceptibility. The SM HST victorin produced by *Cochliobolus victoriae* likely targets host proteins that are monitored by LOV1 in *Arabidopsis thaliana*. In oat, the victorin susceptibility *Vb* locus is linked to the *Pc-2* locus that confers resistance to the obligate biotrophic rust pathogen *Puccinia coronata* f. sp. *avenae*. Likely, this rust fungus secretes an *Avr* protein that is directly or indirectly recognized by Pc-2 that mediates PCD.

2008a, b). Also *Tsn1* encodes an NBS-LRR receptor protein containing an S/T protein kinase that is required for ToxA sensitivity and disease susceptibility (Faris *et al.*, 2010). This NBS-LRR protein is very similar to the RPG5 stem rust resistance protein active against *P. graminis* (Brueggeman *et al.*, 2008). Thus, NBS-LRRs can be involved in HST-triggered susceptibility (ETS) to necrotrophs and ETI to biotrophs. Many NBS-LRR resistance proteins active against biotrophs do not interact directly with cognate fungal effectors but guard he host targets of these effectors. When effectors of biotrophic pathogens interact with host targets, this is sensed by the guarding resistance proteins that subsequently mediate PCD (Jones & Dangl, 2006). A similar guard model may also hold for necrotrophic plant–pathogen interactions. Tsn1 is an NBS-LRR-like host protein that does not interact directly with ToxA but might guard the host target of ToxA (Faris *et al.*, 2010).

Thus, both proteinaceous effectors of biotrophic fungal pathogens and SMs or proteinaceous HSTs of necrotrophic fungal pathogens interact with host targets that are guarded by (CC)-NBS-LRR receptor proteins that mediate PCD. Necrotrophic, in contrast to (hemi-) biotrophic, pathogens are tolerant to harsh conditions prevalent in necrotic tissues (Lorang *et al.*, 2007). Is (CC)-NBS-LRR-mediated PCD induced by HSTs different from PCD induced by biotrophic proteinaceous effectors and how is resistance then achieved against necrotrophs? Some plants resistant to necrotrophs might lack a target for a HST. Indeed such plants are resistant to infections by a necrotroph, as is for example the case for *C. heterostrophus* where absence of URF13 in the host leads to insensitivity to T-toxin. But for (hemi-) biotrophic and necrotrophic pathogens producing proteinaceous HSTs like ToxA and the SnToxs (Friesen *et al.*, 2008a, b; Ciuffetti *et al.*, 2010) the situation might be different. Several common signaling pathways are associated with both ETS against necrotrophs and ETI against (hemi-)biotrophs. Thus, a classic gene-for-gene relationship proposed for biotrophs might also hold true for some (hemi-)biotrophs and some necrotrophs producing proteinaceous toxins, whereas the inverse gene-for-gene relationship might only hold true for a subgroup of necrotrops. Biotrophs and hemi-biotrophs cannot thrive on dead host tissue likely because they are not equipped with the appropriate weaponry to survive in necrotic tissues, whereas necrotrophs are equipped with the suited weaponry to colonize necrotic tissues. It is expected that future research will identify important biochemical components of this latter weaponry as more and more genomes of *Dothideomycete* fungi become available.

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