Dothideomycete effector facilitating biotrophic and necrotrophic lifestyles

Thierry T. Rouxel, Pierre U.G.M. de Wit

EFFECTORS IN PLANT-MICROBE INTERACTIONS

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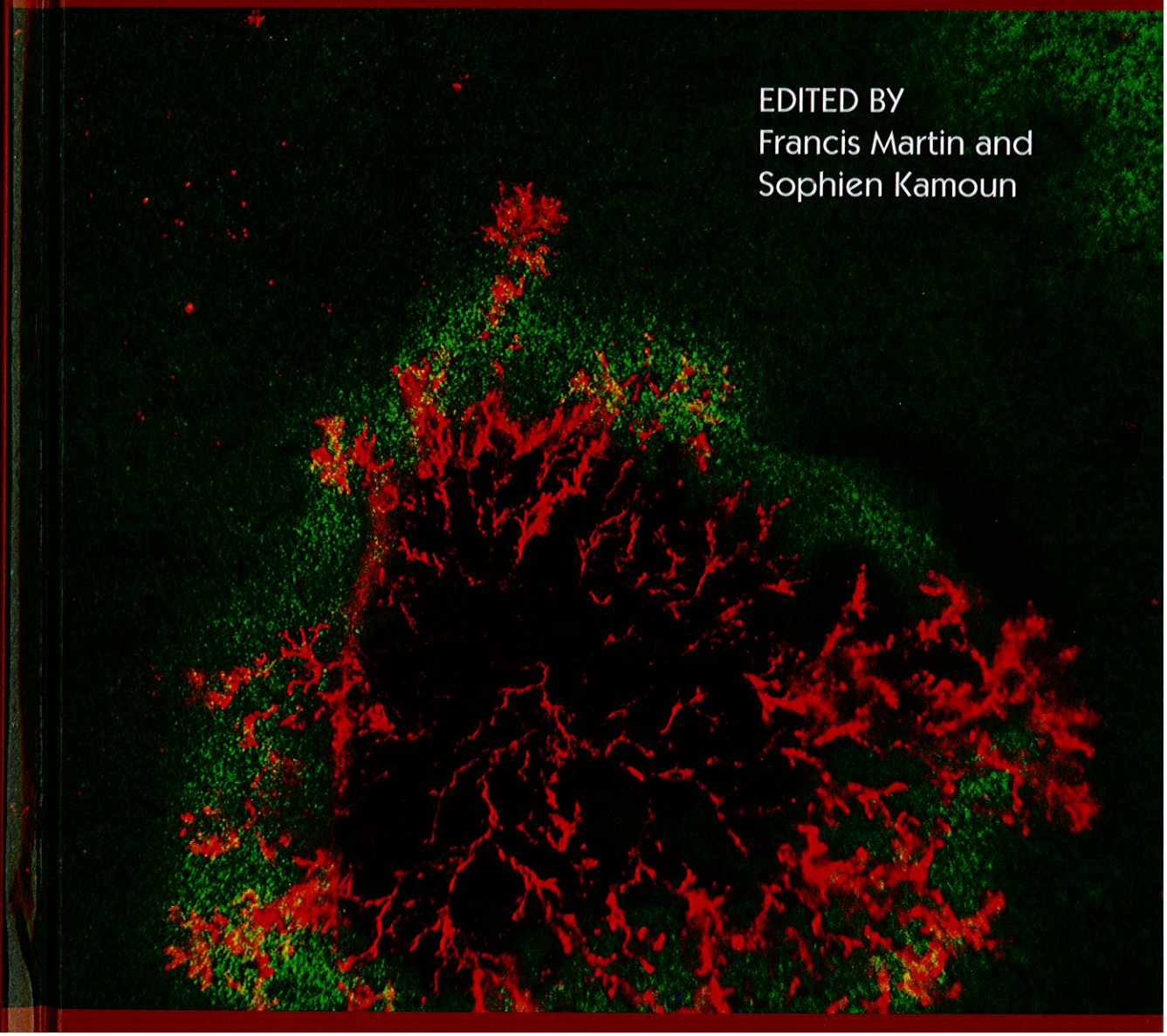

8 Dothideomycete Effectors Facilitating Biotrophic and Necrotrophic Lifestyles

Thierry Rouxel and Pierre J.G.M. de Wit

8.1 Introduction to *Dothideomycetes*

8.1.1 Phylogeny and Ecology

Dothideomycetes represent the largest and phylogenetically the most diverse class of fungi within the *Ascomycota* with 1300 genera and over 19,000 known species (Kirk et al., 2008; Schoch et al., 2009). Very diverse ecological niches are inhabited by *Dothideomycetes* representing many different lifestyles. Most *Dothideomycetess* are saprobes, but they also contain lichens, coprophilous fungi, endophytes, and plant pathogens (Schoch et al., 2009). Terrestrial fungi, including rock-inhabiting, fresh water and marine species are also found within *Dothideomycetes* (Schoch et al., 2009). A similar diversity of lifestyles and niches is found at the order level, as exemplified by the diversity found within the *Pleosporales* (Zhang et al., 2009) (Fig. 8.1). Plant pathogens are often confined to a number of terminal clades, always residing within saprobic lineages, strongly suggesting that pathogenicity to plants (or endophytic behavior) is a secondary acquisition departing from saprobes. A maximum of seven independent transitions to pathogenicity is suggested to have occurred in lineages of the orders *Pleosporales*, *Capnodiales* (Fig. 8.1), *Myriangiales*, and *Botryosphaeriales* and the family of *Venturiaceae* (Schoch et al., 2009). Within the *Pleosporales*, the well-documented family of *Pleosporineae* contains the majority of plant pathogens (e.g., the genera *Cochliobolus*, *Phaeosphaeria*, *Leptosphaeria, Pyrenophora*, and *Dydimella*) that diverged from a common ancestor more than 64 million years ago (Zhang et al., 2009). The *Capnodiales* also contain many plant pathogens (e.g., the genera *Passalora* and *Mycosphaerella*) (Fig. 8.1). Other well-known plant pathogenic *Dothideomycetes* belong to the genera *Venturia* and *Botryosphaeria* infecting crop plants, vegetables, or tree species.

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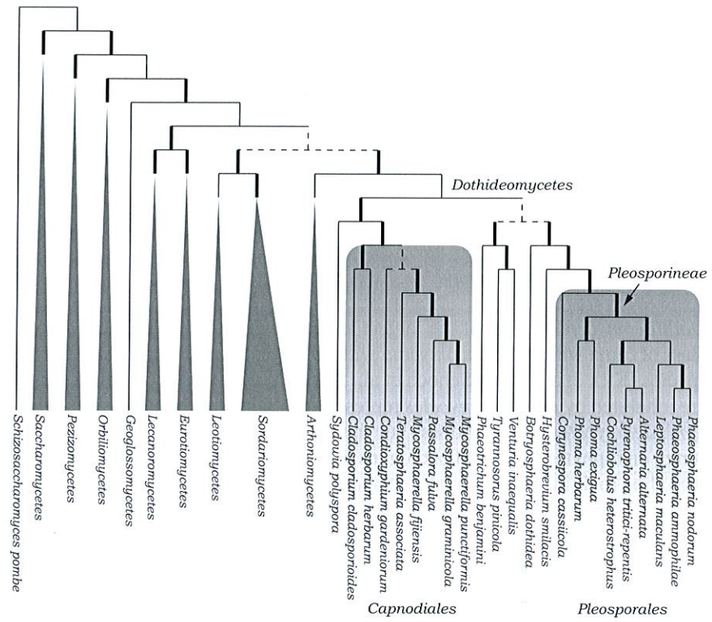

Fig. 8.1 A phylogenetic tree of major lineages in Ascomycota with a selection of plant pathogenic lineages in *Dothideomycetes*. The phylogenetic analysis was done using RaxML. Classes outside of the *Dothideomycetes* were collapsed in TreeDyn. Thick branches on the tree represent nodes that had more than 70% bootstrap values in a RAxML run. Stippled lines represent nodes that had less than 50% bootstrap values (C. Schoch, personal communication).

Some of them have become model species to study several aspects of host–pathogen interactions including:

(a) Avirulence (*Avr*) genes. The first fungal *Avr* gene ever cloned was isolated from the Dothideomycete *Passalora fulva* (*Cladosporium fulvum*) that became a model (de Wit, 2007; de Wit and Spikman, 1982; van Kan et al., 1991; Joosten and de Wit, 1999). More recently, additional *Avr* genes have been cloned from a second *Dothideomycete* species, *Leptosphaeria maculans* (Fudal et al., 2007; Gout et al., 2006; Parlange et al., 2009).
(b) Genes encoding proteinaceous effectors or enzymes involved in the production of secondary metabolites acting as host-selective toxins (HST) have been isolated from the *Pleosporineae* (see Chapter 15).

(c) Whole-genome searches for effectors have been initiated for several *Dothideomycete* species, including *L. maculans*, *C. fulvum*, *Mycosphaerella graminicola, Mycosphaerella fijiensis*.

8.1.2 Modes of Pathogenesis

Although *Venturia* species penetrate the cuticle and thrive subcuticularly during the biotrophic phase (McHardy, 1996), the plant pathogenic *Dothideomycetes* are in general extracellular pathogens that grow on the plant surface, penetrate plants through natural openings like stomata and grow in the extracellular space (also known as the apoplast). Representative for such extracellular growth is *P. fulva* and species of the genera *Cochliobolus*, *Leptosphaeria*, *Mycosphaerella, Pyrenophora*, and *Phaeosphaeria*. Alditol sugars such as mannitol, which can only be metabolized by the fungi themselves (Solomon et al., 2003, 2006, 2007), often accumulate in the apoplast. In the apoplast, the fungi do not produce feeding structures like haustoria. Thus, they must manipulate the host from the outside in order to retrieve nutrients. Not much is known about how nutrients are retrieved from the host, but in general the pathogens can have a biotrophic, hemibiotrophic, or necrotrophic lifestyle likely reflecting their mode of nutrient retrieval. Biotrophs manipulate their host in a subtle way keeping host cells alive for a prolonged time, whereas necrotrophs likely thrive on dead cells. It is assumed that proteinaceous and metabolite effectors play an important role in determining these different lifestyles. Some necrotrophic fungi, like *Pyrenophora* sp. and *Phaeosphaeria* sp. produce necrogenic peptides that kill host cells (Friesen et al., 2008a). The necrotic response is dependent on host targets for these peptides (Friesen et al., 2008b).

8.1.3 Genome Initiatives in Dothideomycetes

Ten *Dothideomycete* genomes have been sequenced so far, of which three are published, and several others are in progress, (Hane et al., 2007; Goodwin et al., 2011; Rouxel et al., 2011; Table 8.1). The first sequenced genomes (*Phaeosphaeria* (*Stagonospora*) *nodorum*, *M. graminicola*, *Pyrenophora tritici-repentis*, and *Alternaria brassicicola*; Table 8.1) are compact with only few transposable elements (TEs) as is often the case for fungal genomes. However, the genome of *L. maculans* is more complex: one-third of the genome consists of TEs (Table 8.1). This phenomenon seems to be true for other sequenced *Dothideomycete* genomes. Indeed, *M. fijiensis* (Table 8.1), *C. fulvum* (de Wit, unpublished data) and *Venturia inaequalis* (Le Cam, unpublished data) seem to have undergone massive TE invasion with more than half of the genome made up of TEs. In the case of *L. maculans*, the TE invasion is recent

Table 8.1 Features of genomes of a few *Dothideomycetes*.

	Leptosphaeria maculans	*Phaeosphaeria (Stagonospora) nodorum*	*Pyrenophora tritici-repentis*	*Cochliobolus heterostrophus*	*Alternaria brassicicola*	*Mycosphaerella fijiensis*	*Mycosphaerella graminicola*
No. of chromosomes	17–18	19	11	15–16	9–11	?	21[a]
Genome size (Mb)	45.1	36.6	37.8	34.9	30.3	73.4	39.7
No. of contigs	1743	496	703	400	4039	5883	21
No. of SuperContigs (SCs)	77	107	47	89	838	382	21
SC L50 (Mb)	1.8	1.1	1.9	1.3	2.4	2.0	Finished
Gaps (%)	2.5	0.4	1.7	1.1	5.4	8.9	0.01
No. of predicted genes	12,469	10,762	12,141	9633	10,688	10,327	10,952
Average gene length (bp)	1323	1326	1618	1836	1523	1627	1600
GC content (%)	44.1	50.3	50.4	52–54	50.5	nd	55.0
Repeat content (%)	34.2	7.1	16.0	7.0	9.0	50.0	18.0
"Core" genome size (Mb)[b]	29.7	34.5	31.7	32.5	27.6	36.7	32.6
Gene density/core genome (no of gene per 10 kb)	4.2	3.1	3.8	3.0	3.9	2.8	3.4

Genome data are at http://urgi.versailles.inra.fr/index.php/urgi/Species/Leptosphaeria (*L. maculans*), http://www.broad.mit.edu/annotation/genome/stagonospora_nodorum/Home.html (*P. nodorum*) (Hane et al., 2007), http://www.broadinstitute.org/annotation/genome/pyrenophora_tritici_repentis/Home.html (*P. tritici-repentis*), http://genome.jgi-psf.org/CocheC5_1/CocheC5_1.home.html (*C. heterostrophus*), http://genome.jgi-psf.org/Altbr1/Altbr1.home.html (*A. brassisicola*), http://genome.jgi-psf.org/Mycfi1/Mycfi1.home.html (*M. graminicola*).

[a] Variable number due to the existence of variable number of dispensable chromosomes.

[b] "Core" genome excluding the repeated elements, but including the gaps in the genome sequence.

and took place after the separation from other species within the *Pleosporineae* (Rouxel et al., 2011).

8.2 Pregenome Identification of Avirulence and Effector Genes in *Dothideomycetes*

8.2.1 Avr gene Products or Elicitors

In gene-for-gene interactions, pathogen avirulence (*Avr*) gene products elicit a resistance response in plants that carry a cognate resistance (*R*) gene, which is usually associated with a hypersensitive response (HR). It is assumed that the Avr protein triggers the cognate plant R protein that subsequently mediates downstream defense signaling pathways, culminating in HR and phytoalexin accumulation. For this reason, Avr proteins were previously called elicitors (Keen, 1975).

Thus, elicitors were initially identified by their ability to trigger *R* gene-mediated HR and accumulation of phytoalexins. Since Flor coined the gene-for-gene hypothesis, many pathologists have studied different pathosystems to find experimental evidence for the gene-for-gene hypothesis by molecular and biochemical approaches. In 1984, Flor's gene-for-gene hypothesis could be proven experimentally by cloning the first bacterial *Avr* gene (Staskawicz et al., 1984).

In 1991, the first fungal *Avr* gene was cloned from *C. fulvum* (van Kan et al., 1991) and the first oomycete *Avr* gene from *Phytophthora sojae* followed in 2004 (Shan et al., 2004).

8.2.2 Avr Genes of C. fulvum

To date, four *Avr* genes have been cloned from *C. fulvum* that all encode small cysteine-rich proteins secreted during infection, including Avr2, Avr4, Avr4E, and Avr9, whose recognition in tomato is mediated by the cognate Cf (for *C. fulvum*) proteins Cf-2, Cf-4, Cf-4E, and Cf-9, respectively (de Wit et al., 2009; Joosten and de Wit, 1999; Thomma et al., 2005). Additional extracellular proteins (Ecps), Ecp1, Ecp2, Ecp4, and Ecp5 have been characterized from this fungus that cause HR on tomato accessions carrying a cognate *Cf-Ecp* gene (de Kock et al., 2005; Laugé et al., 2000). Recently, Ecp6 and Ecp7 have been identified, but for these two effectors no HR-responding tomato accessions have been identified yet (Bolton et al., 2008). All Avrs and Ecps are assumed to be virulence factors (Bolton et al., 2008; Thomma et al., 2005; van Esse et al., 2007, 2008).

The Avr2 effector inhibits some tomato cysteine proteases, including Rcr3, Pip1, aleurain, and TDI-65, that are presumed to be important in basal host

defense (Krüger et al., 2002; Rooney et al., 2005; Shabab et al., 2008; van Esse et al., 2008). *Botrytis cinerea* and *Verticillium dahliae* are more virulent on *Arabidopsis thaliana* transgenics expressing *Avr2* compared to control *A. thaliana* (van Esse et al., 2008) indicating that Avr2 also facilitates virulence of other fungal tomato pathogens. In the presence of Cf-2, Avr2 behaves as an avirulence factor and its recognition is mediated by *Rcr3^{pimp}* (required for *C. fulvum* resistance), a cysteine protease originating from *Lycopersicon pimpinellifolium* (Krüger et al., 2002; Luderer et al., 2002b; Rooney et al., 2005). Structural modification of Rcr3 by Avr2, rather than Rcr3 inhibition, is the most likely cause of triggering Cf-2-mediated defense signaling. Indeed, in the presence of Cf-2, a natural variant of Rcr3 occurring in *Lycopersicon esculentum* (Rcr3^{esc}) causes spontaneous HR in an Avr2-independent manner, although it is still an active enzyme. The Rcr3^{esc} protein is likely to have a modified tertiary structure as compared to the Rcr3^{pimp} protein (Krüger et al., 2002).

The apoplast contains antimicrobial compounds, including enzymes such as chitinases and proteases, and secondary metabolites against which the fungi need to be protected. The Avr4 effector contains a functional chitin-binding domain that protects chitinous fungi, including *C. fulvum*, against plant chitinases (van den Burg et al., 2003, 2004, 2006, van Esse et al., 2007). In the presence of Cf-4, Avr4 induces an HR. However, some natural isoforms of Avr4 can bind chitin without triggering Cf-4-mediated HR (Joosten et al., 1997; Stergiopoulos et al., 2007; van den Burg et al., 2003).

The virulence function of Avr4E is not known yet, but in the presence of Cf-4E, Avr4E triggers Cf-4E-mediated resistance (Westerink et al., 2004).

The Avr9 effector contains a cystine knot domain with structural but so far no functional homology to carboxypeptidase inhibitors (van den Hooven et al., 2001; Vervoort et al., 1997). Disruption of *Avr9* in *C. fulvum* did not affect virulence on tomato plants, suggesting that *Avr9* is not required for full virulence (Marmeisse et al., 1993). However, *Avr9*-expressing tomato plants appear to be more susceptible to natural *C. fulvum* strains that lack *Avr9*, suggesting that Avr9 is a virulence factor with redundant activity (H.P. van Esse and B.P.H.J. Thomma, personal communication). Expression of *Avr9* in vitro is induced under nitrogen-limiting conditions (Pérez-Garcia et al., 2001; Thomma et al., 2006; van den Ackerveken et al., 1994). *C. fulvum* mutants deleted of the *Nrf1* gene (for nitrogen responsive factor) do no longer express *Avr9* under nitrogen-limiting conditions in vitro and are compromised in their virulence on Cf-0 tomato plants. However, these strains are still avirulent on Cf-9 tomato plants, suggesting that Nrf1 is a major, but not the only, positive regulator of *Avr9* expression (Pérez-Garcia et al., 2001). Other *Avr* and *Ecp* effector genes of *C. fulvum* and *L. maculans Avr* and effector genes are not induced under nitrogen-limiting conditions (Bolton and Thomma, 2008; Thomma et al., 2006; Rouxel et al., 2011), indicating that induction by nitrogen-starvation is a specific feature of the *Avr9* effector gene.

8.2.3 Avr *Genes of* L. maculans

At least 11 distinct *Avr* genes, designated *AvrLm1* to *AvrLm11*, have been genetically mapped in *L. maculans* (Balesdent et al., 2002; A. Degrave and M.H. Balesdent, unpublished data). The initial genetic mapping indicated clustering for a series of *Avr* genes such as *AvrLm1*, *AvrLm6*, and *AvrLm2* (Balesdent et al., 2002) or *AvrLm4*, *AvrLm7*, and *AvrLm3* (Balesdent et al., 2005). Such unusual genetic clustering of *Avr* genes has been recently observed in another *Dothideomycete*, *V. inaequalis,* with two main clusters encompassing (i) *AvrVdg2*, *AvrVv1*, *AvrVu1*, and *AvrVrjrd*; and (ii) *AvrVu2*, *AvrVh3.2*, *AvrVs1*, and *AvrVu4* (Broggini et al., 2011). Sequencing of the *L. maculans* genome revealed an unusual structure. The genome is bipartite, with distinct GC-equilibrated and AT-rich blocks also termed isochores. The two types of genomic regions have different recombination frequency and gene content. The AT-rich isochores comprise one-third of the genome but only contain 5.0% of the predicted protein coding sequences and multiple mosaics of inactivated and more or less truncated TEs. Strikingly, they contain all three *Avr* genes cloned to date, but are likely to contain other currently mapped *Avr* genes. The absence of meiotic recombination also explains why genetic clusters of *Avr* genes represent hundreds of kb in AT-rich genomic regions (Fudal et al., 2007). The three cloned *Avr* genes, *AvrLm4–7*, *AvrLm1*, and *AvrLm6* are single copy genes, encoding small secreted proteins (SSPs) that have no or few homologs in databases. In contrast to AvrLm1, AvrLm6 and AvrLm4–7 contain six to eight cysteine residues, resembling the cysteine-rich effectors of *C. fulvum* and *Fusarium oxysporum* f. sp. *lycopersici*, and might be secreted in the apoplast (Gout et al., 2006; Parlange et al., 2009).

AvrLm4–7 confers avirulence on both *Rlm7* and *Rlm4* genotypes (Parlange et al., 2009). In field populations, only three of the four possible allele combinations are found, the double virulent haplotype (*a4a7*), the double avirulent haplotype (*A4A7*), and the a4A7 haplotype giving virulence on *Rlm4-* but avirulence on *Rlm7-* containing cultivars (Parlange et al., 2009). Large-scale sequencing of alleles, along with targeted mutagenesis experiments indicated that a single base pair change in the *A4* allele, resulting in a single amino acid change in the protein (glycine to arginine) was sufficient to evade *Rlm4-*mediated resistance (Parlange et al., 2009). This residue has shown to be located on an external loop of the protein, strongly suggesting that the altered recognition is not due to a change in the overall fold of the mature protein (I. Fudal, F. Blaise, K. Blondeau, and H. van Tilbeurgh, unpublished data).

Comparison of virulent and avirulent isolates of *L. maculans* on fields of susceptible oilseed rape plants, and comparison of near-isogenic isolates in controlled conditions both indicated a strong fitness penalty linked with the lack of avirulence function at the *AvrLm4–7* locus, whereas the effect was much lower or not measurable at the *AvrLm1* locus (Huang et al., 2006, 2010). These data suggest that AvrLm4–7, and to a much lesser extent AvrLm1, are virulence factors. The strong involvement of AvrLm4–7 in virulence is consistent with the finding that in nature, and in the absence of the *Rlm7* selection, no virulent strains are found that lack *AvrLm4–7* (Balesdent et al., 2006).

8.2.4 Ecp *Effectors of* C. fulvum

Effector genes coding for Ecps have been cloned from *C. fulvum*, including *Ecp1, Ecp2, Ecp4, Ecp5, Ecp6*, and *Ecp7* (Bolton et al., 2008; Laugé et al., 2000; van den Ackerveken et al., 1993). Ecps are abundantly secreted by all strains of *C. fulvum* during infection and possess an even number of cysteine residues most likely involved in intramolecular disulfide bridges (Luderer et al., 2002a). *Ecp1, Ecp2*, and *Ecp6* are all virulence genes, since their silencing or disruption compromises virulence of *C. fulvum* on tomato (Bolton et al., 2008; Laugé et al., 1997). Tomato accessions have been identified carrying single cognate dominant *Cf-Ecp* genes mediating HR triggered by *Ecp1*, *Ecp2*, *Ecp4*, and *Ecp5*. However, these *Cf-Ecp* genes have not been cloned yet (Laugé et al., 2000; Laugé et al., 1998; Soumpourou et al., 2007).

Orthologs of Ecp6 are detected in many other fungal species mainly because they contain CBM14 and LysM-domains, implicated in carbohydrate binding including chitin. This suggests that Ecp6 might be a functional homolog of Avr4 (Bolton et al., 2008). However, Ecp6 does not protect fungi against plant chitinases but is involved in scavenging chitin fragments released from fungal cell walls during infection, thus preventing them from acting as pathogen-associated molecular patterns (PAMPs, that trigger pathogen-triggered immunity) (de Jonge et al., 2010).

8.2.5 Effectors from L. maculans *and Other* Dothideomycetes

Effectors were sought in *V. inaequalis* by generating a cDNA library during a compatible interaction. The library was probed with genomic DNA from *V. inaequalis* and cDNA libraries from in vitro-grown fungus to identify fungal cDNAs (Bowen et al., 2009). A suppression subtractive hybridization library enriched for cellophane-induced genes was also generated as fungal growth on cellophane triggers differentiation of structures similar to what is observed in planta. Sixteen new candidate effector genes were identified from *V. inaequalis* with features common to characterized effector genes from other filamentous fungi (encoding small, novel, cysteine-rich proteins with a putative signal peptide). Three of the 16 candidate effectors genes showed significant induction during growth in planta (Bowen et al., 2009).

In *L. maculans,* small-scale sequencing of the AT-rich genomic regions surrounding *AvrLm1* and *AvrLm6* identified two additional candidate effector genes termed *LmCys1* and *LmCys2* (Fudal and Profotova, unpublished data). Functional analyses, including silencing of expression or complementation, strongly suggest a role for *LmCys2* in virulence of *L. maculans* and confirm that AT-rich isochores host genes involved in virulence or avirulence in this species. *LmCys1* shows similarity with *FoSIX1* of *F. oxysporum* and its silencing affects *L. maculans* growth in vitro (Fudal and Profotova, unpublished data). *LmCys1* is thus postulated to be involved in virulence. However, neither transformation of *F. oxysporum* with *LmCys1* nor transformation of *L. maculans* with *FoSIX1* had a measurable effect on virulence (I. Fudal and M. Rep, unpublished data).

8.3 Pregenomic Identification of Host-Selective Proteinaceous Toxins of *Dothideomycetes*

8.3.1 Proteinaceous Toxins

Obligate and biotrophic fungal pathogens retrieve their nutrients from living host cells, whereas necrotrophic pathogens thrive on killed host cells. In biotrophic pathosystems, a dominant pathogen gene product can trigger resistance on a host carrying a matching resistance gene, whereas necrotrophic pathogens produce proteinaceous effectors (some also known as host-selective toxins) promoting disease and the host produces receptors required for susceptibility. Therefore, necrotrophic pathosystems can be seen as a mirror image of the classical gene-for-gene interaction (Friesen et al., 2008a).

P. nodorum and *P. tritici-repentis* are two necrotrophic fungal pathogens that produce several necrogenic host-specific peptide effectors that can be recognized by host susceptibility genes to cause disease.

P. nodorum is a fungal pathogen of wheat causing the *Stagonospora nodorum* blotch (SNB) disease. SnTox1 was the first toxic peptide produced by *P. nodorum* shown to interact with a corresponding host susceptibility gene, *Snn1* (Friesen et al., 2006, 2007; Liu et al., 2006). SnToxA is encoded by a gene highly similar to the Ptr *ToxA* gene from *P. tritici-repentis*, the causal agent of tan spot of wheat with the matching susceptibility gene *Tsn1*. Tsn1-disrupted mutants were insensitive to both Ptr ToxA and SnToxA, suggesting that both toxins are functionally similar as they are recognized by the same

locus in the host. Therefore, the Tsn1–ToxA interaction in the wheat-*P. nodorum* pathosystem parallels that of the wheat-tan spot system, and the wheat *Tsn1* gene serves as the major determinant for susceptibility to both SNB and tan spot (Liu et al., 2006).

The *SnTox2–Snn2* interaction was the third gene pair identified in this system. SnTox2 is a small secreted peptide of about 7 kDa. Sensitivity to SnTox2 is conferred by the single dominant gene *Snn2*. In contrast to the classical gene-for-gene model, the *Tsn1–SnToxA* and the *Snn2–SnTox2* interactions are additive in their contribution to susceptibility (Friesen et al., 2007).

SnTox3 is the fourth necrosis-inducing peptide, triggering Snn3-dependent necrosis. However, although the SnToxA–Tsn1 and SnTox2–Snn2 interactions have additive effects, both the SnToxA–Tsn1 and SnTox2–Snn2 interactions are epistatic to the SnTox3–Snn3 interaction (Friesen et al., 2008b; Zhang et al., 2009).

ToxA was the first peptide toxin produced by the most common races of *P. tritici-repentis*. Ptr ToxA, is a 13.2-kDa protein causing necrosis in particular genotypes of wheat (Ciuffetti et al., 1997). ToxA interacts with a chloroplast ToxA binding protein 1 (ToxABP1) of wheat mesophyll cells, through its RGD-containing, solvent-exposed loop. This results in ToxA internalization, leading to cell death (Manning et al., 2008). ToxABP1 contains a lysine-rich region within a coiled-coil domain similar to phosphatidyl-inositol binding sites present in animal proteins involved in endocytosis. ToxABP1 protein is present in both chloroplast membranes and chloroplast stroma. Surprisingly, *ToxABP1* is expressed at similar levels and encodes an identical protein in both ToxA-sensitive and -insensitive cultivars, indicating that ToxA should have other targets besides ToxABP1 (Manning et al., 2007).

Other *Dothideomycetes* also produce small-size proteinaceous toxins such as the AB-toxin of *A. brassicicola*, the ABR-toxin of *Alternaria brassicae*, and the AP-toxin of *Alternaria panax* (Lawrence et al., 2008; Parada et al., 2008). These generally cause symptoms in host, but not in nonhost plants and are postulated to be HSTs (Parada et al., 2008). They are produced during infection suggesting that they are effectors (Otani et al., 1998).

8.3.2 Allergens of Alternaria Species

Alternaria alternata spores are one of the most common and potent airborne sources of allergens (Lawrence et al., 2008). Currently, eight proteinaceous allergens have been identified in *A. alternata*, including the major allergen Alta1 (Thaker et al., 1995; Yunginger and Jones, 1978). *Altb1*, a highly conserved homolog of *Alta1*, is expressed by *A. brassicicola* in vitro and highly upregulated during *A. thaliana* infection (Cramer and Lawrence, 2003, 2004). Over 52 species of *Alternaria* and very closely related fungi possess highly

conserved *Alta1* homologs suggesting that virtually every species within the genus is potentially allergenic (Hong et al., 2005). A highly homologous gene is present in *L. maculans* and found in EST libraries of infected plant tissue (Rouxel et al., 2011). Other minor *Alternaria* sp. allergen homologs, such as *Alta5* and *Alta6*, are present in the genomes of other *Dothideomycetes*, such as *L. maculans*. Their expression is also upregulated at the onset of plant infection (Rouxel et al., 2011). Alta1 possesses phosphatase activity suggesting that it has phytotoxic properties with a putative function in virulence (Lawrence et al., 2008). The increased expression of *L. maculans Alta1*, *Alta5*, and *Alta6* homologs during plant infection suggests that they act as effectors. However, not all these proteins show the typical features of effectors: only Alta5 and Alta6 contain a signal peptide, indicating they may be secreted in the apoplast, they are not enriched in cysteine residues, and do not reside in the AT-rich isochores of *L. maculans* genome but in GC-equilibrated regions.

8.4 Whole-Genome Searches for Effectors

8.4.1 General Facts

Recently, whole-genome sequencing of fungal pathogens has generated an enormous amount of data that can be screened for putative (secreted) effectors. Enrichment for effector candidates can be achieved by integration of genome, transcriptome, proteome, and metabolome data, when available. Comparative secretome analysis and basic local alignment search tool (BLAST) sequence similarity searches could also be used to identify putative effectors in sequenced genomes, but it will only prove successful when sufficient homology exists among effector genes such as seen for Avr4, Ecp6, or the functional homolog of ToxA protein from *P. nodorum* in *P. titici-repentis* (Friesen et al., 2008a; Friesen et al., 2006). In addition, secretome approaches require protocols to induce effector secretion in vitro, which are often lacking (Vincent et al., 2009). One main drawback of genome-wide searches for effector genes lies in the automated annotation process that is often insufficient to correctly identify small-sized genes with low or no homology with known genes or domains. One elegant way to address this problem is to search for effector genes located in TE-rich genomic regions. Precise annotation of TE families enables the extraction of nonrepeated regions that can subsequently be screened for effector genes. In the case of *Dothideomycetes*, this approach was successfully used in *L. maculans* (Rouxel et al., 2011). Despite the great potential of genome-wide searches for candidate effector genes, the function of these genes still needs to be confirmed experimentally by overexpression, gene disruption, or silencing in fungal isolates and subsequent (a)virulence assays on host plants.

8.4.2 *The Search for Orthologs of* C. fulvum *Effectors in Other* Dothideomycete *Fungi*

Most fungal effectors characterized so far are species-specific and facilitate virulence on a particular host. During infection of its host plant tomato, *C. fulvum* secretes effectors that function as virulence factors in the absence of cognate Cf resistance proteins. Whole-genome searches identified homologs of the *C. fulvum* Avr4 and Ecp2 effectors in other *Dothideomycete* species, including *M. fijiensis*, the causal agent of the black sigatoka disease of banana (Stergiopoulos et al., 2010). The Avr4 homolog of *M. fijiensis* was shown to be a functional ortholog of *C. fulvum* Avr4 that protects fungal cell walls against hydrolysis by plant chitinases through binding to chitin and, despite its low sequence homology, triggers a Cf-4-mediated HR in tomato. Furthermore, three homologs of the *C. fulvum* Ecp2 were found in *M. fijiensis,* one of which induces different levels of necrosis or HR in tomato lines lacking or not a putative cognate Cf-Ecp2 protein. In contrast to Avr4 that acts as a defensive virulence factor, the *M. fijiensis* Ecp2 likely promotes virulence by interacting with a putative host target causing host cell necrosis. Cf-Ecp2 could possibly guard the virulence target of Ecp2, triggering an HR. Avr4 and Ecp2 represent core effectors that are recognized by single cognate Cf-proteins. Transfer of these *Cf* genes to plant species attacked by fungi containing those cognate core effectors, could provide new opportunities for disease resistance breeding.

V. inaequalis causes scab of apple and other members of the Maloideae. Although a member of the *Dothidiomycetes*, its precise placement in this class is not yet clear. A comprehensive sequence analysis places it outside the subclass and order (Schoch et al., 2009; Fig. 8.1). A first draft of the *V. inaequalis* whole-genome sequence reveals the presence of effectors homologous to effectors of other *Dothidiomycete* pathogens (Bowen et al., 2011). *V. inaequalis* contains an ortholog of *Ecp6* from *C. fulvum* with all three LysM domains and shares 50% identity at the amino acid level. It also contains a large gene family of over 30 members with moderate homology (30% identity at the amino acid level) to the AvrLm6 effector from *L. maculans*.

8.4.3 *Whole-Genome Searches for Effectors in the* L. maculans *Genome*

In contrast to what is observed for the *C. fulvum* effectors, the currently known *Avr* and effector genes from *L. maculans* have no recognizable homologs, or exhibit only very weak similarity with effectors of other *Dothideomycete* species (Parlange et al., 2009; I. Fudal and B. Profotova, unpublished data). In *L. maculans*, candidate effector genes were specifically sought by examination of genes within AT-rich isochores, hypothesized to be specific niches for these genes. As described above, AT-isochores carry few genes. Protein comparisons

and gene ontology analysis indicated that AT-isochores are enriched in genes having potentially a key role in virulence (Rouxel et al., 2011). These include orphan genes encoding SSPs, genes involved in response to chemical or biotic stimuli, as well as nonribosomal peptide synthetases and polyketide synthases involved in biosynthesis of secondary metabolites. In the AT-isochores, ~20% of the genes (122 genes) encode putative SSPs versus only 4.2% of the genes (529 genes) in the GC-isochores. SSP-encoding genes from AT-isochores have features indicative of effectors such as a low GC content, low EST support, low expression in vitro and induction during plant infection, lack of recognizable domains or homologs in other fungi, and a high cysteine content. They do not have paralogs and only two of them (1.8%) had a best BLAST match to a predicted protein from *P. nodorum*. By contrast, putative SSPs from GC-isochores do not show effectors features, in particular 110 (20.8%) hit to hypothetical proteins from *P. nodorum* using BLAST (Rouxel et al., 2011).

8.4.4 *Whole-Genome Search for Effectors in the* M. graminicola *Genome*

To date, the *M. graminicola* genome has only been mined for putative effector-encoding genes through an unusual criterion (Rudd et al., 2010). The authors postulated that, similarly to what is found in some bacterial effectors, fungal effectors may contain internal tandem repeats that may change their interaction specificity. Twenty-three protein-encoding genes predicted to be secreted and containing internal tandem repeats were found, half of which is overexpressed during plant infection. In addition, most of them showed repeat number polymorphisms from one isolate to the other. There is no definite proof yet that they act as effectors but this work might open new ways to discover novel candidate effector genes in genomes of other members of the *Dothideomycetes*.

8.5 Translocation of Fungal Effectors

Fungal effector proteins can be roughly grouped into extracellular effectors, secreted into the apoplast or xylem of the host plant and act extracellularly, and cytoplasmic effectors that are (i) either secreted into the apoplast and subsequently translocated into host cells or (ii) directly translocated into host cells. Despite the low degree of sequence conservation among fungal effectors, most of them code for SSPs, of which some are postulated to be translocated into host cells by a yet unknown mechanism. Extracellular effectors are often *N*- and sometimes *C*-terminally processed, as shown for the Avr and Ecp effectors of *C. fulvum* (Stergiopoulos and de Wit, 2009) and the SIX effectors of *F. oxysporum* f. sp. *lycopersici* (Houterman et al., 2007). Another common feature of many fungal (and *Dothideomycete*) effectors is the presence of

multiple cysteine residues that might be involved in disulfide-bridge formation providing protein stability. Some effectors are active inside the host cell like those of rusts and possibly need proper folding and disulfide-bridge formation outside the host before being taken up (Dodds et al., 2004; Kemen et al., 2005). The cytoplasmic or nuclear localization of the cognate R proteins directed against rusts, powdery mildews, *Magnaporthe oryzae* and *F. oxysporum* f. sp. *lycopersici* or plant protein targets of their effectors suggest that these effectors are likely translocated into these compartments (Catanzariti et al., 2007; Dodds et al., 2004, 2006; Ellis et al., 2007; Houterman et al., 2009; Jia et al., 2000; Shen et al., 2007). Oomycete plant pathogens are known to produce a wide variety of effectors that are delivered in the plant cell using translocation signals composed of an RXLR motif and a nearby acidic dEER motif (Rehmany et al., 2005; Tyler et al., 2006; Jiang et al., 2008). These allow interaction with phosphatidylinositides, thus mediating internalization of the effector-lipid complex by a yet unknown mechanism (Kale et al., 2010). Although fungal effectors known to date do not contain RXLR or dEER motifs, it has been suggested that variants of these motifs can be functional (Dou et al., 2008). On the basis of this, a bioinformatic pipeline was set up to propose a probability score to predict whether commonly found <[RKH] × [LMIFYW]> or <[RKH] [LMIFYW] × [RKH]> motifs could act as oomycete RXLR motifs. Apart from occurrence of the motifs, other criteria included presence of a signal peptide, location of the putative motif from start of the protein sequence, and composition of the motifs (Rouxel et al., 2011). The pipeline was applied to the complement of *L. maculans* putative effectors and showed that 60% of SSPs in AT-isochores have putative "RXLR-like" motifs, identifying these SSPs as candidate cell-entering effectors (Rouxel et al., 2011). At present, we have no information on cell location of R proteins corresponding to the known Avr proteins of *L. maculans*. However, these predicted motifs were found to be functional in a series of cases: along with a few other fungal avirulence proteins; *L. maculans* AvrLm6 was shown to possess two putative translocation motifs in its *N*-terminus (RTLK and RYWT), of which the RYWT but not the RTLK was found to be functional to allow Pi3P binding and internalization in plant or animal cells (Kale et al., 2010). Similarly, the AvrLm4–7 protein contains two putative translocation motifs in its *N*-terminus, KGLI and RAWG, of which the solvent-exposed RAWG motif but not the other one was functional (S. Kale and B. Tyler, unpublished data; Chapter 10). These data indicate that many *Dothideomycete* (and fungal) effectors might be internalized following their secretion in the apoplast, and that degenerated RXLR motifs are present allowing binding of Pi3P and translocation of the complex into the plant cell. An alternative signal for translocation is found in the host-selective protein toxin ToxA of *P. triticirepentis* (Ciuffetti et al., 1997). ToxA contains a solvent-exposed Arg-Gly-Asp (RGD) motif that interacts with the host plasma membrane and is likely

required for its internalization (Manning et al., 2008). A similar RGD motif mediating interaction with the plasma membrane is also present in the IpiO (AvrBlb1) effector protein of the Oomycete *Phytophthora infestans* (Senchou et al., 2004; Vleeshouwers et al., 2008) and Ecp5 of *C. fulvum* (Laugé et al., 2000).

8.6 Effector Diversification and Avoidance of R Protein-Mediated Resistance

8.6.1 Birth and Diversification of Effectors

Little is known of the mechanisms governing birth and diversification of effectors in *Dothideomycete* species. In some cases, conservative evolution is observed with effector-encoding genes found in a series of related species (see Section 8.4.2). In other cases, such as the *ToxA* gene, the birth of an effector gene might be ascribed to horizontal gene transfer (HGT), a phenomenon occurring in *Pezizomycotina* (Oliver and Solomon, 2008). Similarly, some *Avr* and effector gene products of *L. maculans* have weak homology with effectors from divergent species such as *F. oxysporum* (e.g., LmCys1 with Six1/Avr3), but not with other *Dothideomycetes*, which may indicate HGT.

In *L. maculans*, very few of the ca. 50 putative effector-encoding genes located in AT-rich isochores have identified orthologs in related species of the suborder *Pleosporineae* (see Section 8.4.3). Effector genes have been reported to reside in specific regions of the genome of some eukaryotic genomes. Some effector genes of *M. oryzae* are subtelomeric (Farman, 2007), or associated with telomere-like repeats in protozoan parasites of animals, such as *Plasmodium* and *Trypanosoma* species (Pain et al., 2008). Genomes of many *Fusarium* species contain supernumerary linkage-specific chromosomes enriched in strain-specific effectors that account for the host range of each "*forma specialis*" (Ma et al., 2010; Coleman et al., 2009). These linkage-specific chromosomes can translocate from pathogenic to nonpathogenic *Fusarium* species. Similarly, toxin-encoding genes, including proteinaceous effectors, of *Alternaria* species are often "B" chromosome-borne (Lawrence et al., 2008). The genome of *P. infestans* has a plethora of effector candidates embedded in repetitive DNA and diversification of these effectors is postulated to occur via segmental duplication and intraspecific copy number variation resulting in rapidly diverging multigene families (Haas et al., 2009). The association between the AVR_{k1} family of effectors and a TE in *Blumeria graminis*, the barley powdery mildew fungus, is proposed to provide a mechanism for amplification and diversification of this effector family (Sacristan et al., 2009). Diversification of effectors in the species mentioned above is postulated to be associated with TE-driven gene duplication and generation of multigene

families. Similarly, SSP-encoding genes of *L. maculans* mostly associate with three types of DNA transposons, representing a minor part of the TEs in the genome (Rouxel et al., 2011). In contrast to the examples mentioned above, duplicated effector genes have not been identified in *L. maculans* so far. This is consistent with the steady inactivation of TEs by repeat-induced point mutation (RIP) and with an ancient TE activity prior to their RIPping. This could suggest an accelerated diversification mechanism of ancestral gene(s) that no longer allows recognition of orthologs in related species.

8.6.2 Adaptation of Effectors to R*-Gene Selection Pressure*

In *C. fulvum*, bypassing of the Avr-triggered Cf-mediated HR can be achieved by point mutations, deletions, or transposon insertions in the *Avr* genes (Luderer et al., 2002b; Stergiopoulos et al., 2007).

Evasion of Avr2 recognition is mainly achieved by frameshift mutations or transposon insertions in the *Avr2* gene. This leads to less virulent strains. Evasion of Cf-4-mediated recognition is mainly achieved by single point mutations in the *Avr4* gene leading to instable Avr4 proteins (Joosten et al., 1997). However, these proteins can still protect the fungus against deleterious effects of chitinases as they can still bind to chitin (Stergiopoulos et al., 2007). Evasion of Cf-4E-mediated recognition is achieved by point mutations in the *Avr4E* gene or jettison of the *Avr4E* gene, suggesting that the fitness penalty associated with the loss of this gene is not very high (Stergiopoulos et al., 2007). However, *Avr4E*-expressing tomato plants are more susceptible to natural *C. fulvum* strains that lack *Avr4E* than control plants, suggesting that Avr4E is a virulence factor (H.P. van Esse and B.P.H.J. Thomma, personal communication).

Cf-9-mediated defense is avoided by strains carrying *Avr9* by jettison of the gene. No other mutations leading to virulence on Cf-9 plants have been observed so far for this gene, suggesting that Avr9 is not a crucial virulence factor (van den Ackerveken et al., 1992). However, overexpression of Avr9 in Cf-0 tomato plants increases virulence to *C. fulvum* races lacking the *Avr9* gene, indicating that Avr9 might have a virulence function (H.P. van Esse and B.P.H.J. Thomma, personal communication).

In the case of *L. maculans*, occurrence of *AvrLm* genes in a TE-rich genome environment, that inhibits meiotic recombination, seems to be detrimental for allele diversification. Accordingly, complete deletion of the *AvrLm1* gene and the surrounding 260-kb region is the most common mode of evolution toward virulence on *Rlm1* plants reported (Gout et al., 2007). This deletion event disseminated among field populations during the 3 years following the commercial release of *Rlm1* oilseed rape cultivars (Rouxel et al., 2003). A different situation was observed in the *AvrLm4–7* avirulence gene: a single nonsynonymous base mutation, suppressing recognition by the Rlm4 resistance protein without

altering recognition by Rlm7, is the only adaptation toward virulence reported (Parlange et al., 2009). In between these two extremes, bypassing of the *Rlm6*-mediated resistance was associated with multiple events, including complete or partial deletions (66% of the cases) and numerous cases of point mutations (Fudal et al., 2009). Most of the point mutations could be attributed to RIP that mutated 4–9% of bases in the coding sequence, generating 2–4 stop codons, and alternate splice sites (Fudal et al., 2009).

8.7 Concluding Remarks

The *Dothideomycetes* comprise a large group of fungi, including biotrophic, hemibiotrophic, and necrotrophic plant pathogens that colonize a wide range of different plant species. Most of them penetrate leaves through stomata and thrive in the apoplastic space where they secrete various types of effectors that facilitate virulence. Intramolecular disulfide bridges stabilize many effector proteins in the apoplastic environment. Recent data suggest that some of the *Dothideomycete* effector candidates bind to phosphatidylinositides of the host membrane through RXLR-like motifs, facilitating internalization into the plant cell. Future research will investigate whether this is a general mechanism used by many *Dothideomycete* species.

Effectors of *Dothideomycetes* have functions that reflect their different lifestyles. Just like effectors of biotrophic fungal pathogens, toxic peptides from necrotrophic pathogens interact with host targets. However, the effectors of necrotrophic pathogens cause necrosis facilitating disease and might therefore be considered as basic effectors for which no true gene-for-gene-based resistance has been developed yet, due to a low level of coevolution. One could envisage that host targets of effectors of necrotrophic and biotrophic pathogens are intrinsically similar. However, biotrophic pathogens effectors would have coevolved with their host target, and no longer mediate necrosis but manipulate host targets in a more subtle way (de Wit et al., 2009; Stergiopoulos et al., 2010; Fig. 8.2). Future research on more divergent *Dothideomycetes* might provide support to this hypothesis.

Comparative genome analyses performed on *Dothideomycetes* revealed that these fungi use various strategies to generate effectors and to adapt to changing environments. Comparison to other fungal classes showed several cases of massive (and probably recent) genome invasions by TEs. These are likely to favor lateral effector gene transfer, transposon-induced changes, and RIP acting on effector genes. These phenomena sometimes combined with sexual recombination provide these fungi with the tools to adapt quickly to changing environments. The link between TE invasion, effector birth and diversification, speciation, and adaptation to new environments (including new host plants) provides fascinating topics for future research.

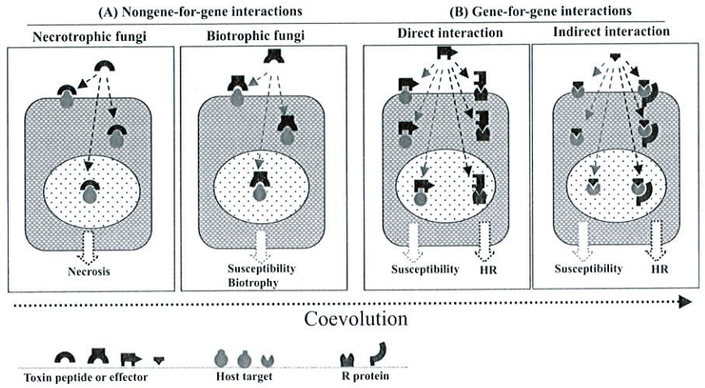

Fig. 8.2 Interactions between effectors of necrotrophic and biotrophic fungi and their host targets. **(A)** Nongene-for-gene interactions. Necrotrophic fungi: Effectors of necrotrophic fungi interact with host targets eventually causing necrosis of host plant cells to facilitate disease. Biotrophic fungi: Effectors of biotrophic fungi interact with host targets and facilitate disease without killing host plant cells. **(B)** Gene-for-gene interactions. It is assumed that as a result of coevolution with their host plants, biotrophic fungi have evolved further. Direct interaction: It is assumed that effectors have two domains of which one interacts with the virulence target leading to host susceptibility, while the other is recognized by the R protein present in resistant plants leading to the hypersensitive response (HR) and resistance. Indirect interaction: The effectors interact with the virulence target leading to susceptibility. Resistant plants have developed resistant proteins that do not interact directly with effectors but guard the virulence target and HR is triggered after sensing manipulation of the virulence target by the effector. Interaction between effectors and host targets (virulence targets and R proteins) can take place on the plasma membrane, in the cytoplasm, or in the nucleus. (Adapted from de Wit et al., 2009.)

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