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Effector proteins of extracellular fungal plant pathogens that trigger host resistance

Ann-Maree Catanzariti[A] *and David A. Jones*[A,B]

[A]Division of Plant Science, Research School of Biology, RN Robertson Building (46), The Australian National University, Canberra, ACT 0200, Australia.
[B]Corresponding author. Email: david.jones@anu.edu.au

Abstract. An understanding of the molecular mechanisms that plant pathogens use to successfully colonise host tissue can be gained by studying the biological activity of pathogen proteins secreted during infection. Several secreted 'effector' proteins with possible roles in virulence have been isolated from extracellular fungal pathogens, including three that have been shown to negate host defences. In most cases, significant effector variation is observed between different pathogen isolates, driven by the recognitional capacity of disease resistance proteins arrayed against the pathogen by the host plant. This review summarises what is known about the expression, function and variation of effectors isolated from extracellular fungal pathogens.

Additional keywords: avirulence, effector-triggered immunity, hypersensitive response, plant disease resistance, virulence.

Introduction

Microbial plant pathogens secrete proteins (effectors) to acquire nutrients and modulate plant defences in order to successfully invade and colonise host tissue. To counter this attack, plants have evolved resistance (R) proteins for the recognition of specific pathogen effectors, resulting in a plant defence response known as effector-triggered immunity (ETI). Thus, effector proteins that have evolved to conduct virulence functions can themselves become recognition factors and, consequently, alteration or loss of effector genes enables pathogens to circumvent detection. This creates an ongoing evolutionary battle of attack and counter-attack. The recognition of effectors by R proteins occurs both directly through physical contact and indirectly via specific changes in the host cell due to the action of the effector (Caplan *et al*. 2008). However, regardless of the mechanism, a recognition event renders the pathogen unable to cause disease and, thus, effectors that activate ETI are termed avirulence (Avr) proteins. This review deals specifically with effector proteins from extracellular fungal pathogens that activate ETI. A summary of these proteins is given in Table 1.

Extracellular fungal pathogens colonise the intercellular spaces of host tissue – either in the apoplast surrounding parenchyma cells or the lumen of xylem vessels – but do not penetrate host cell walls to form feeding structures. Effectors secreted from this group of pathogens have been isolated largely from the apoplastic fluid or xylem sap of infected tissue; their corresponding genes have been cloned by reverse genetics. These effectors are small and generally cysteine-rich, which is consistent with the formation of intramolecular disulfide bonds, which provide stability in protease-rich environments. They are highly expressed *in planta* but often show little or no expression *in vitro* and are frequently unique proteins with no sequence similarity to proteins of known function. Although many of these effectors have been found to confer a fitness advantage, the actual virulence function has been determined for only three effectors and in each case the effector is involved in the suppression or avoidance of host defences.

Cladosporium fulvum

One of the best studied extracellular fungal plant pathogens is the tomato leaf mould fungus *Cladosporium fulvum*. This foliar pathogen penetrates the leaf surface through stomata and grows within the apoplast (Fig. 1). To date, eight *C. fulvum* proteins secreted into the apoplast have been shown to function as avirulence determinants. These have been divided into two groups: (i) race-specific Avr proteins and (ii) extracellular proteins (Ecps), which are found in all strains of *C. fulvum* that have been examined. The Ecp effectors (Ecp1, Ecp2, Ecp4 and Ecp5) are recognised in tomato lines carrying cognate *Cf-Ecp* genes (Laugé *et al*. 1997; Lauge *et al*. 2000; de Kock *et al*. 2005); however, these R genes have not yet been cloned. Each *Ecp* locus appears to be highly monomorphic among *C. fulvum* isolates. This is thought to reflect a lack of selection pressure, given that *Cf-Ecp* genes have not been used in commercial tomato cultivars (Stergiopoulos *et al*. 2007). The function of these Ecp effectors is currently unknown; however, pathogenicity tests showed that mutated strains of *C. fulvum* lacking Ecp1 or Ecp2 were less virulent than their wild-type counterpart (Laugé *et al*. 1997). Recently, three Ecp2 homologues were identified in *Mycosphaerella fijiensis*, a fungus that causes black Sigatoka disease of banana (Stergiopoulos *et al*. 2010). One of these homologues

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Table 1. Effector proteins identified from extracellular fungal plant pathogens that are known Avr determinants

Effector	Protein size (aa)		Cys no.[A]	Function/role in virulence	Reference
	Transcript	Mature			
Cladosporium fulvum					
Avr2	78	58	8	Cysteine protease inhibitor	Luderer *et al.* (2002)
Avr4	135	86	8	Protects against chitinases	Joosten *et al.* (1994)
Avr4E	121	101	6	Unknown	Westerink *et al.* (2004)
Avr9	63	28	6	Unknown	van den Ackerveken *et al.* (1992)
Ecp1	96	65	8	Confers fitness advantage	Laugé *et al.* (1997)
Ecp2	165	143	4	Confers fitness advantage	Laugé *et al.* (1997)
Ecp4	119	101	6	Unknown	Lauge *et al.* (2000)
Ecp5	115	98	6	Unknown	Lauge *et al.* (2000)
Fusarium oxysporum f. sp. *lycopersici*					
Avr1	242	184	6	Suppression of I-2 and I-3 resistance	Houterman *et al.* (2008)
Avr2	163	144	2	Required for full virulence	Houterman *et al.* (2009)
Avr3	284	189	8	Required for full virulence	Rep *et al.* (2004)
Leptosphaeria maculans					
AvrLm1	205	183	1	Confers fitness advantage	Gout *et al.* (2006)
AvrLm4–7	143	122	8	Confers fitness advantage	Parlange *et al.* (2009)
AvrLm6	144	124	6	Unknown	Fudal *et al.* (2007)
Rhynchosporium secalis					
NIP1	82	60	10	Necrosis-inducing toxin	Rohe *et al.* (1995)

[A]Number of cysteine resides in the mature protein.

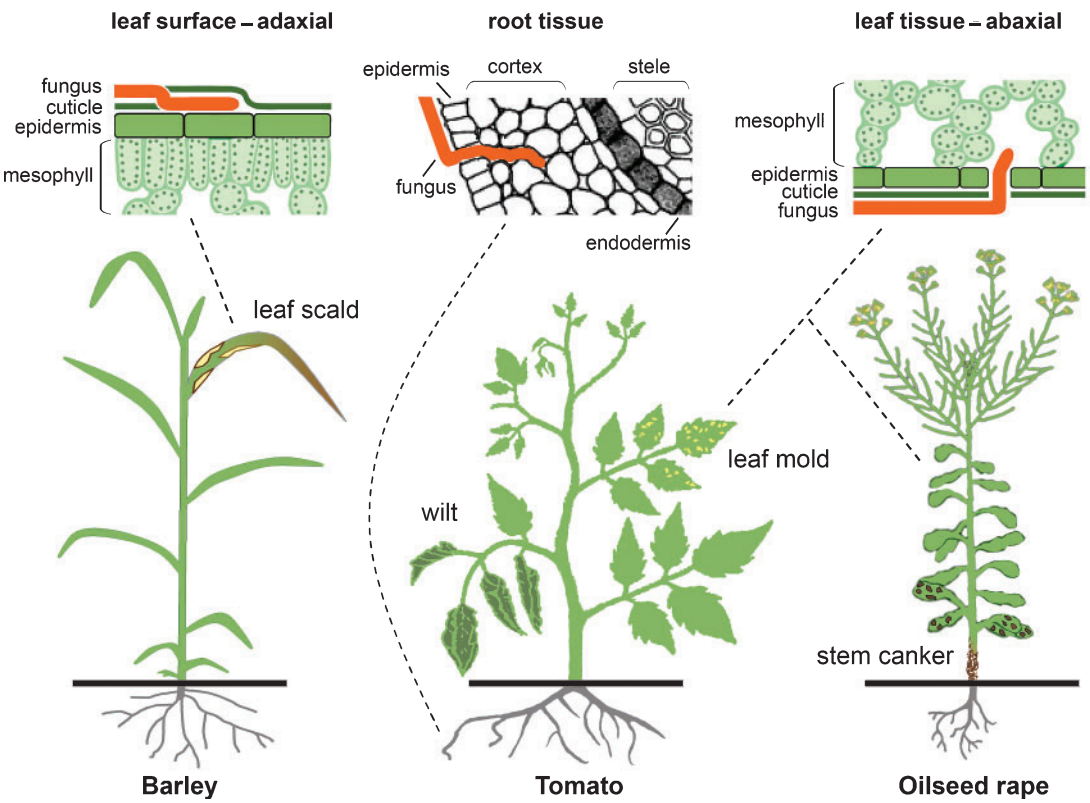

Fig. 1. Infection by extracellular fungal pathogens and sites of effector delivery. Routes of fungal entry and pre-sporulation plant disease symptoms are represented for each of the pathosystems presented in this review. (Left) *Rhynchosporium secalis* grows intercellularly in the subcuticular region of the barley leaf causing scald lesions. (Middle) *Fusarium oxysporum* f. sp. *lycopersici* penetrates through the root cortex, and grows within the xylem vessels (not shown), which results in wilt disease on tomato. (Right) *Cladosporium fulvum* and *Leptosphaeria maculans* both infect through stomata and grow within the leaf apoplast. During later infection stages, *L. maculans* grows within the vascular tissue (not shown) towards the crown, causing stem canker on oilseed rape. *C. fulvum* remains in the apoplast and causes chlorotic spots on tomato, typical of leaf mould disease.

(MfEcp2) was found to be recognised in tomato *Cf-Ecp2* lines, although, MfEcp2 also caused a reduced disease resistance-like response on lines lacking *Cf-Ecp2* (Stergiopoulos *et al*. 2010). Two additional effectors, Ecp6 and Ecp7 have also been identified (Bolton *et al*. 2008), but tomato lines containing a corresponding resistance gene have not yet been reported so these effectors will not be discussed here.

The Avr2, Avr4, Avr4E and Avr9 effectors are recognised in tomato lines carrying *Cf-2*, *Cf-4*, *Cf-4E* and *Cf-9*, respectively (van den Ackerveken *et al*. 1992; Joosten *et al*. 1994; Luderer *et al*. 2002; Westerink *et al*. 2004). Each of these R genes encodes a membrane-anchored protein with extracellular leucine-rich repeats (LRRs), although the exact recognition mechanisms leading to ETI are not known. Two of these effectors, Avr2 and Avr4, have virulence functions that suppress and avoid host defences, respectively.

Avr2 is a 58 amino acid (aa) cysteine protease inhibitor that inhibits several tomato proteases involved in basal host defence (Rooney *et al*. 2005; Shabab *et al*. 2008; van Esse *et al*. 2008). A virulence function is reinforced by the observation that *Arabidopsis* expressing *Avr2* is more susceptible to the extracellular fungal pathogens *Botrytis cinerea* and *Verticillium dahliae* (van Esse *et al*. 2008). The recognition of Avr2 by the Cf-2 protein is indirect and requires the cysteine protease Rcr3. During infection, Avr2 binds and inhibits Rcr3 and this interaction, which is thought to cause a conformational change to Rcr3, triggers Cf-2-mediated resistance (Rooney *et al*. 2005). Strains of *C. fulvum* that evade recognition by Cf-2 have altered *Avr2* genes containing transposon insertions, gene deletions or mutations encoding amino acid changes (Luderer *et al*. 2002).

Avr4 is produced as a preproprotein that is both *C*- and *N*-terminally cleaved after secretion to a mature protein of 86 aa. This effector binds chitin to protect the fungal cell wall against the effects of plant chitinases (van den Burg *et al*. 2006). Silencing *Avr4* significantly reduced *C. fulvum* virulence, whereas tomato expressing *Avr4* is more susceptible to *C. fulvum* and other fungal pathogens of tomato (van Esse *et al*. 2007). Most variants of Avr4 that do not trigger Cf-4 resistance have mutations affecting cysteine residues involved in disulfide bonds, resulting in an unstable protein that is more susceptible to proteolysis (van den Burg *et al*. 2003). Consequently, these unstable variants do not accumulate in the apoplast or elicit ETI, but they do still provide protection against plant chitinases by binding chitin in the fungal cell wall, an association that protects them against degradation by apoplastic proteases (van den Burg *et al*. 2003). Like Ecp2, a homologue of Avr4 was recently identified in *M. fijiensis* (MfAvr4), which was also shown to be a functional orthologue (Stergiopoulos *et al*. 2010). The MfAvr4 protein binds chitin to protect fungal cell walls against plant chitinases and is also recognised in tomato lines carrying *Cf-4*. The Avr4 and Ecp2 effectors may therefore represent important proteins required for pathogenicity on a range of hosts.

Avr4E is a 101 aa secreted protein of unknown function. *C. fulvum* strains virulent on *Cf-4E* tomato lines have either lost the *Avr4E* gene or have point mutations that encode a stable Avr4E protein with two single amino acid alterations, with only one of these amino acid changes being required to circumvent Cf-4E-mediated resistance (Westerink *et al*. 2004). Avr9 is secreted as a preproprotein that is *C*- and *N*-terminally cleaved to a mature protein of 28 aa. Avr9 contains three disulfide bridges that form a cysteine knot but, despite structural homology to the carboxypeptidase inhibitor, the function of Avr9 remains unknown (Vervoort *et al*. 1997; van den Hooven *et al*. 2001). *C. fulvum* strains virulent on tomato carrying *Cf-9* lack the *Avr9* gene, and *Avr9* knockout lines exhibit no loss in virulence (Marmeisse *et al*. 1993). *Avr9* is induced *in vitro* under low nitrogen conditions and is regulated by the *Nrf1* gene (Pérez-García *et al*. 2001). *Nrf1* knockouts show reduced virulence on susceptible tomato, but are still avirulent on *Cf-9* plants, suggesting Nrf1 is not the only regulator of Avr9. No other *Avr* or *Ecp* effector genes are induced under nitrogen-limiting conditions and their regulators are currently unknown (Thomma *et al*. 2006).

Fusarium oxysporum *f. sp.* lycopersici

The soil-borne fungal pathogen *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) infects roots of tomato plants via wounds or by direct penetration and colonises xylem vessels causing wilt disease (Fig. 1). Seven proteins have been isolated from xylem sap during *Fol* infection – termed secreted in xylem (Six) proteins. The recent genome sequencing of *Fol* has revealed that the *SIX* genes are all located on the same chromosome, which is absent from non-pathogenic *F. oxysporim* (Ma *et al*. 2010). This chromosome was experimentally shown to be responsible for pathogenicity on tomato by transferring it to a non-pathogenic strain, converting it to a pathogen (Ma *et al*. 2010). Three Six proteins function as Avr determinants in tomato cultivars carrying the corresponding R genes. After secretion, Avr3 (Six1) is cleaved at both the *N*- and *C*-terminus to yield a 22 kDa (189 aa) protein and a 12 kDa derivative, the former being the form thought to trigger resistance in tomato cultivars carrying the *I-3* resistance gene (Rep *et al*. 2004; M. Rep, pers. comm.). Expression of *Avr3* requires the presence of living plant cells and occurs immediately upon penetration of the root cortex (van der Does *et al*. 2008). The exact function of this effector is currently not known; however, it is required for full virulence and a loss of *Avr3* causes a significant fitness penalty (Rep *et al*. 2005). Accordingly, all natural *Fol* isolates contain *Avr3* and no sequence variants that overcome *I-3* recognition have been found (Rep *et al*. 2004, 2005). Nonetheless, *Fol* isolates virulent on *I-3* cultivars do exist and overcome resistance by the presence of a second effector protein, Avr1, which inhibits I-3-mediated resistance (Houterman *et al*. 2008).

The Avr2 (Six3) effector is 144 aa and contains just two cysteine residues (cf. other effectors, see Table 1). The cognate *I-2* gene, currently the only *Fol* resistance gene to be cloned, encodes an intracellular nucleotide-binding leucine-rich repeat (NB-LRR) protein with an *N*-terminal coiled coil (CC) domain (Simons *et al*. 1998). Recognition of Avr2 occurs inside the plant cell (Houterman *et al*. 2009), consistent with the location of I-2, although it is not known whether these two proteins directly interact. Furthermore, an intracellular recognition implies that Avr2 is translocated from the xylem into the host cell, a feature that is common to effectors from biotrophic fungi and oomycetes that penetrate the host cell wall and form membrane-invaginating

feeding structures (Catanzariti *et al*. 2007; Panstruga and Dodds 2009). Avr2 is also required for full virulence in susceptible hosts and no isolates have been found with deletions of the *Avr2* gene. Variants of Avr2 carrying single amino acid changes have been found that circumvent I-2-mediated resistance with no apparent fitness cost (Houterman *et al*. 2009). Nonetheless, inhibition of I-2-mediated resistance, also mediated by Avr1, is a more frequent mechanism underlying virulence on *I-2* cultivars (Houterman *et al*. 2008).

Avr1 (Six4) is processed after secretion and is present in the xylem sap as a 184 aa mature protein (Houterman *et al*. 2008). *Fol* strains carrying Avr1 trigger a resistance response in tomato cultivars carrying either the *I* or the *I-1* gene but is not required for virulence on plants lacking the cognate *I* genes (Houterman *et al*. 2008). Conversely, on *I-2* and *I-3*, plants, Avr1 functions as an inhibitor of ETI, although the mechanism of this inhibition is not yet known. No variation in the Avr1 sequence has been detected among different *Fol* isolates and the *Avr1* gene is absent from strains avirulent on *I-2* or *I-3* plants (Houterman *et al*. 2008).

Leptosphaeria maculans

The stem canker fungus *Leptosphaeria maculans* causes blackleg disease on brassicas (Fig. 1). During the symptomless phase of the disease, the fungus penetrates the leaf through stomatal openings and grows within the mesophyll layer before entering the vascular tissue and moving into the base of the stem. Map-based cloning has been used to identify three *L. maculans* avirulence genes, *AvrLm1* (Gout *et al*. 2006), *AvrLm6* (Fudal *et al*. 2007) and *AvrLm4–7* (Parlange *et al*. 2009), which are recognised by yet to be isolated *Rlm* resistance genes in oilseed rape (*Brassica napus* L.). All three *Avr* genes encode small proteins with predicted signal peptides and have similar expression profiles, with the highest level of expression seen during leaf infection and a low level of expression *in vitro* (Fudal *et al*. 2007; Parlange *et al*. 2009). Unlike AvrLm6 and AvrLm4–7, AvrLm1 contains only one cysteine residue and, therefore, may be targeted to the cytosol of the host cell, analogous to *Fol* Avr2 and the cysteine-poor effectors from biotrophic fungi and oomycetes that are known to enter host cells (Catanzariti *et al*. 2007). Most isolates that have overcome *Rlm1*-mediated resistance carry a deletion of the *AvrLm1* gene (Gout *et al*. 2007). Nevertheless, a measurable fitness cost has been reported for virulence alleles at the *AvrLm1* locus, suggesting that this effector does have a virulence function (Huang *et al*. 2010). Similarly, a significant fitness advantage is associated with an intact *AvrLm4–7* gene suggesting that the AvrLm4–7 effector also has a virulence function (Balesdent *et al*. 2006; Huang *et al*. 2006, 2010). This effector confers avirulence on both *Rlm4* and *Rlm7 B. napus* lines, and appears to have dual recognition specificity. Evaluation of natural isolates virulent on *B. napus* carrying *Rlm4* or *Rlm7* found that evasion of *Rlm7*-mediated resistance was associated with deletion of the *AvrLm4–7* gene, whereas evasion of *Rlm4*-mediated resistance was frequently the result of a single amino acid change in the AvrLm4–7 protein, suggesting different recognition mechanisms (Parlange *et al*. 2009). However, this amino acid change is also associated with a reduced fitness (Parlange *et al*. 2009).

Rhynchosporium secalis

The fungus *Rhynchosporium secalis* is the causal agent of leaf scald on barley (Fig. 1). After penetrating the cuticle, *R. secalis* grows intercellularly in the subcuticular region of the host leaf where it secretes three small proteins that function as non-specific toxins (Wevelsiep *et al*. 1991). These proteins were purified from fungal culture filtrates and when injected into the leaves of barley and other non-host cereals, these toxins, termed necrosis-inducting proteins (NIP), cause scald-like lesions. NIP1 has a stimulatory effect on the host's plasma membrane H^+-ATPase, which is the likely cause of leaf necrosis (Wevelsiep *et al*. 1993) and is also an avirulence determinant, activating ETI in barley cultivars carrying the *Rrs1* resistance gene (Hahn *et al*. 1993; Rohe *et al*. 1995). NMR spectrometry has revealed a novel protein fold with five disulfide bridges that make NIP1 highly stable (van't Slot *et al*. 2003). Most races of *R. secalis* virulent on *Rrs1* cultivars lack the *Nip1* gene, but some have alleles with point mutations that generate amino acid changes (Schürch *et al*. 2004). Sequence variants of this effector, which do not induce *Rrs1*-mediated resistance, also lack necrosis-inducing activity, suggesting that NIP1 function and Rrs1 recognition are inherently linked. Furthermore, NIP1 binds a single plasma membrane receptor, found in susceptible and resistant cultivars, which mediates both necrosis and *Rrs1*-dependent resistance (van't Slot *et al*. 2007).

Concluding remarks

The identification of numerous novel secreted effector proteins from extracellular fungal pathogens suggests they conduct diverse functions during infection. The presence of even numbers of cysteine residues and their occurrence in intercellular wash fluids suggests these effectors function outside the host cell. Indeed, this is true for the *C. fulvum* effectors Avr2 and Avr4, which inhibit plant apoplastic proteases and bind chitin, respectively. However, this may not be the case for all effectors, particularly AvrLm1, which has only one cysteine residue and would, therefore, be vulnerable to apoplastic proteases, and the *Fol* effector Avr2, which only has two cysteine reidues and activates ETI from inside the plant cell. It is also possible that the *Fol* effector Avr1 functions within host cells as this effector inhibits I-2-mediated resistance triggered by Avr2, although the nature of this inhibition is not known and could act outside the cell to prevent Avr2 uptake. The discovery of fungal effectors that negate plant defences, and the fact that many other effectors have a virulence function, highlights effectors as key components in pathogenicity. Thus, identifying complete pathogen secretomes along with functional studies and the identification of host targets will significantly advance our knowledge of the molecular mechanisms pathogens use to manipulate plant cells to facilitate colonisation and ultimately cause disease. Furthermore, understanding how recognition of effectors by cognate R proteins can be compromised is important not only for understanding the evolution of pathogenicity and plant resistance but also the durability of R genes. As a more complete picture of the molecular mechanisms involved in R-Avr interactions is gathered, better approaches to the use of R genes in the field and the development

of novel disease control mechanisms will undoubtedly become apparent.

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