Pathogen profile

***Venturia inaequalis*: the causal agent of apple scab**JOANNA K. BOWEN^{1,*}, CARL H. MESARICH^{1,2}, VINCENT G. M. BUS³, ROBERT M. BERESFORD¹, KIM M. PLUMMER⁴ AND MATTHEW D. TEMPLETON¹¹The New Zealand Institute for Plant & Food Research Limited, Mt. Albert Research Centre, Private Bag 92 169, Auckland 1142, New Zealand²School of Biological Sciences, University of Auckland, Private Bag 92 019, Auckland 1142, New Zealand³The New Zealand Institute for Plant & Food Research Limited, Hawke's Bay Research Centre, Private Bag 1401, Havelock North 4157, New Zealand⁴La Trobe University, Department of Botany, Bundoora, Vic. 3086, Australia**SUMMARY**

The fungus *Venturia inaequalis* infects members of the Maloideae, and causes the disease apple scab, the most important disease of apple worldwide. The early elucidation of the gene-for-gene relationship between *V. inaequalis* and its host *Malus* has intrigued plant pathologists ever since, with the identification of 17 resistance (*R*)–avirulence (*Avr*) gene pairings. The *Avr* gene products are presumably a subset of the total effector arsenal of *V. inaequalis* (predominantly proteins secreted *in planta* assumed to facilitate infection). The supposition that effectors from *V. inaequalis* act as suppressors of plant defence is supported by the ability of the pathogen to penetrate the cuticle and differentiate into large pseudoparenchymatous structures, termed stromata, in the subcuticular space, without the initiation of an effective plant defence response. If effectors can be identified that are essential for pathogenicity, the corresponding *R* genes will be durable and would add significant value to breeding programmes. An *R* gene cluster in *Malus* has been cloned, but no *V. inaequalis* effectors have been characterized at the molecular level. However, the identification of effectors is likely to be facilitated by the resolution of the whole genome sequence of *V. inaequalis*.

Taxonomy: Teleomorph: *Venturia inaequalis* Cooke (Wint.); Kingdom Fungi; Phylum Ascomycota; Subphylum Euascomycota; Class Dothideomycetes; Family Venturiaceae; genus *Venturia*; species *inaequalis*. Anamorph: *Fusicladium pomi* (Fr.) Lind or *Spilocaea pomi* (Fr.).

Life cycle: *V. inaequalis* is a hemibiotroph and overwinters as pseudothecia (sexual fruiting bodies) following a phase of saprobic growth in fallen leaf tissues. The primary inoculum consists of ascospores, which germinate and penetrate the cuticle. Stromata are formed above the epidermal cells but do not penetrate them. Cell wall-degrading enzymes are only produced late in the infec-

tion cycle, raising the as yet unanswered question as to how *V. inaequalis* gains nutrients from the host. Conidia (secondary inoculum) arise from the upper surface of the stromata, and are produced throughout the growing season, initiating multiple rounds of infection.

***Venturia inaequalis* as a model pathogen of a woody host:** *V. inaequalis* can be cultured and is amenable to crossing *in vitro*, enabling map-based cloning strategies. It can be transformed readily, and functional analyses can be conducted by gene silencing. Expressed sequence tag collections are available to aid in gene identification. These will be complemented by the whole genome sequence, which, in turn, will contribute to the comparative analysis of different races of *V. inaequalis* and plant pathogens within the Dothideomycetes.

INTRODUCTION

Venturia inaequalis Cooke (Wint.) is a hemibiotrophic fungus that is the causal agent of the apple disease scab, more commonly known as black spot in Australasia (MacHardy, 1996). *V. inaequalis* has a wide geographical range and is found in almost all areas in which apples are grown commercially. It is the most important disease of apple worldwide in terms of economic cost of control (Carisse and Bernier, 2002). However, the disease is more severe in temperate countries with cool, moist climates during early spring (MacHardy, 1996; Manktelow *et al.*, 1996). Apple scab is predominantly controlled by a combination of sanitation and cultivation measures, and judicious fungicide application, the correct timing of which is essential for effective control (Beresford and Manktelow, 1994). To date, the deployment of resistant cultivars has been very limited because of their relatively poor fruit quality. However, they are expected to play a major role in disease control, particularly in organic fruit production, once new cultivars with durable resistance to scab become available.

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V. inaequalis has been researched continuously for over 100 years. The resultant body of knowledge is comprehensive in its scope, covering aspects of the basic biology and genetics of the fungus, and the epidemiology and control of the disease (reviewed extensively by MacHardy, 1996). This research has revealed the uncommon parasitic strategy adopted by *V. inaequalis*: no host cell penetration occurs, but fungal biomass accumulates in the subcuticular space prior to sporulation. This raises the question as to how the fungus remains undetected in the plant with the absence of a co-ordinated host resistance reaction. The fungus, in all likelihood, produces effectors that suppress any resistance response. Effectors are proteins or secondary metabolites expressed *in planta* that are assumed to facilitate infection/suppress resistance (Chisholm *et al.*, 2006; Dangl and Jones, 2001), some of which have been commandeered by resistance (*R*) genes for pathogen recognition and the initiation of defence. The putative production of effectors by *V. inaequalis* is supported by genetic data.

The fungus is heterothallic and controlled crosses can be performed readily *in vitro*. This led to in-depth genetic studies of *V. inaequalis* in the 1940s and 1950s, when loci for colony colour (presumably involved in melanin biosynthesis), mating type, various nutritional markers and avirulence were identified, and genetic maps were produced (Boone, 1971; Boone and Keitt, 1957; Keitt and Langford, 1940; Shay and Hough, 1952; Williams and Shay, 1957). These studies led to the elucidation of the gene-for-gene nature of the relationship between *V. inaequalis* and *Malus* at the time at which Flor (1956) reported such interactions for the flax-*Melampsora lini* pathosystem. Thus, the *V. inaequalis*-*Malus* pathosystem appears to fit the effector paradigm.

In some pathosystems, effectors are secreted into the apoplast and exert their effect extracellularly, whereas, in others, they are targeted to the host cytoplasm. Generally, effectors that have been identified to date share little amino acid sequence similarity with each other or with other known proteins. Some have limited conserved sequence motifs, e.g. the 'RXLR' or 'ink' motifs of many oomycete effectors (Haas *et al.*, 2009; Schornack *et al.*, 2009). *V. inaequalis* effectors remain elusive and have yet to be cloned (Bowen *et al.*, 2009; Brogginini *et al.*, 2007, 2009a; Kucheryava *et al.*, 2008; Win *et al.*, 2003). Their identification, concomitant with the identification of *R* genes in the host (Belfanti *et al.*, 2004; Boudichevskaya *et al.*, 2009; Brogginini *et al.*, 2009b; Galli *et al.*, 2010b; Malnoy *et al.*, 2008; Szankowski *et al.*, 2009; Vinatzer *et al.*, 2001; Xu and Korban, 2002), is the focus of much of the current molecular research on apple scab disease. Tools for the dissection of the *V. inaequalis*-*Malus* interaction at the molecular level are available. The pathogen can be cultured easily and, as mentioned above, induced to sexually cross *in vitro* (Keitt and Langford, 1941; Keitt and Palmer, 1938).

Although slow growing, it can be transformed readily (Fitzgerald *et al.*, 2003) and is amenable to functional analyses by gene silencing (Fitzgerald *et al.*, 2004). *V. inaequalis* is therefore a tractable, model, hemibiotrophic pathogen of a woody host. Furthermore, assembly of the whole genome sequence (WGS), which is currently underway, will greatly accelerate the molecular dissection of this pathosystem.

TAXONOMY, PATHOGEN EVOLUTION AND HOST RANGE

Debate over the taxonomic classification of many fungal species is often fierce. The case of *V. inaequalis* proves to be no exception. The Venturiaceae, including *V. inaequalis*, has, until recently, been placed in the class Dothideomycetes, in the subclass Pleosporomycetidae, order Pleosporales, on the basis of both morphological and molecular criteria (Eriksson and Hawksworth, 1998; Goodwin, 2004; Lumbsch and Huhndorf, 2007). However, recent comprehensive molecular phylogenetic analyses of members of the Dothideomycetes, using data from both nuclear and mitochondrial genes, place the Venturiaceae outside the Pleosporales, without a subclass and order assignment (Kruys *et al.*, 2006; Schoch *et al.*, 2009).

The classification of the related anamorph (asexual) stage at the genus level has also proven to be controversial. Schubert *et al.* (2003), in the most comprehensive study to date, proposed that the anamorph be classified as *Fusicladium pomi* (Fr.) Lind, replacing the previous classification of *Spilocaea pomi* (Fr.). However, this has not been universally adopted, as the old name of *S. pomi* is still in use (Attrassi *et al.*, 2007; Bini *et al.*, 2008; Jha *et al.*, 2009).

Members of the genus *Venturia* infect various fruit tree genera: *V. inaequalis* infects apple (*Malus*); *V. pirina* infects European pear (*Pyrus*); *V. nashicola* infects Asian pear (*Pyrus*); *V. carpophila* infects peach (*Prunus*); and *V. cerasi* infects cherry (*Prunus*). *Venturia* species can be differentiated into distinct clades on the basis of polymorphisms in ribosomal internal transcribed spacer (ITS) DNA sequences (Beck *et al.*, 2005; Ishii and Yanase, 2000). The topology of the *Venturia* species phylogram aligns closely with that of the host genera, demonstrating a close co-evolutionary relationship between the pathogenic *Venturia* species and their respective fruit tree hosts.

The boundaries between the *Venturia* species are highly distinct and matings between the species in the laboratory have not been achieved. There is evidence of significant variation within each fungal species, presumably as a consequence of annual, sexual reproduction and obligate outcrossing in these heterothallic species (Langford and Keitt, 1957). The species boundaries within a particular host genus, however, are less distinct, e.g. various *Malus* species are able to hybridize, permitting gene flow to occur between these species. It is likely that the resultant

diversity within host genera also adds to the within-species diversity of the *Venturia* species.

Simple sequence repeat (SSR) markers have been used to demonstrate population diversity and to understand the origin and spread of *V. inaequalis* with the domestication of apple worldwide. The domesticated apple is often referred to as *Malus × domestica*, with the '×' in the species binomial denoting that this species resulted from various hybridizations between wild *Malus* species. Coalescence analysis of migration models suggests that *V. inaequalis* originated in Central Asia, the centre of origin of *Malus* spp. (Gladieux *et al.*, 2008, 2010). It is likely that the pathogen followed the dispersal of domesticated apple that accompanied Europeans as they migrated to new territories (Gladieux *et al.*, 2008, 2010). Consistent with this is the observation that there is greater genetic diversity amongst populations of *V. inaequalis* found on *M. siervsii* (the major progenitor of the domesticated apple) in Central Asia than in European populations from *Malus × domestica* and *M. sylvestris* (a secondary crabapple progenitor of the domesticated apple; Gladieux *et al.*, 2010), and also in European subpopulations of *V. inaequalis* than in populations from areas in which apples have been introduced more recently (Gladieux *et al.*, 2008; Tenzer and Gessler, 1997, 1999). In some cases, such as several states in the USA, Japan (Sawamura, 1988) and Australia, *V. inaequalis* introductions have been relatively recent, much later than the introduction of apple itself. Occasionally, in remote growing regions, the disease is absent, e.g. in Western Australia the disease has not become established to date, following continuous eradication efforts after rare and sporadic *V. inaequalis* incursions (McKirdy *et al.*, 2001).

The lack of diversity in both the cultivated hosts and the associated *V. inaequalis* population in regions away from the centre of diversity has resulted in the wild *Malus* species of East Asia remaining as a substantial resource for disease resistance breeding. The majority of disease resistance genes currently being deployed in apple breeding programmes have been sourced from these wild *Malus* species, including those originating from East Asia, e.g. *M. floribunda*, *M. micromalus*, *M. doumeri* and *M. hupehensis*. This is consistent with Nikolai Vavilov's hypothesis that the best germplasm for resistance breeding can be found at the host's centre of origin and diversity, where host and pathogen have co-evolved for the longest period of time (Zhukovsky, 1961).

Certain isolates that can be characterized as *V. inaequalis* on the basis of morphological criteria do not infect apple, but instead infect different hosts belonging to the subfamily Maloideae, such as *Crataegus*, *Sorbus*, *Pyracantha* and *Eriobotrya* species (Biggs, 1990; Le Cam *et al.*, 2002). This indicates either a wide host range for *V. inaequalis* or the need to further subdivide the species into either *formae speciales* or distinct species with separate host specificities. The uncertainty over this issue is,

in part, a result of a lack of a comprehensive host range survey. Le Cam *et al.* (2002) suggested that those isolates responsible for causing scab on *Malus* spp. and *Pyracantha* spp. should be considered as distinct *formae speciales* of *V. inaequalis*, rather than separate species, because they are able to mate with each other *in vitro* and they cannot be differentiated on the basis of polymorphisms in the ITS region of the ribosomal DNA (rDNA) sequences. In contrast, those isolates able to infect *Eriobotrya* should be considered as a separate species because of sequence differences in the ITS region and the gene encoding glyceraldehyde 3-phosphate dehydrogenase (Sánchez-Torres *et al.*, 2009). However, a new study, looking at the variation of six nuclear loci, determined that isolates from the three different hosts did not resolve into three distinct phylogenetic species corresponding to host range (Gladieux *et al.*, 2009), questioning the requirement for novel species delineation and perhaps even subspecific demarcation. Further studies, including WGS comparisons, should clarify the phylogenetic relationships of, and host specificity determinants between, these isolates.

LIFE CYCLE, EPIDEMIOLOGY AND CONTROL ON APPLE

V. inaequalis overwinters predominantly as pseudothecia (sexual fruiting bodies) that develop in apple leaf litter following a phase of saprobic growth after leaf abscission. The fungus is self-sterile, and hence opposite mating types are required for mating to succeed (Keitt and Palmiter, 1937). Some overwintering may also occur as conidial pustules on shoots and bud scales without the involvement of the teleomorph (sexual stage; Becker *et al.*, 1992; Holb *et al.*, 2006). Infection is initiated in spring and early summer by ascospores (sexual spores) that are released by rainfall from pseudothecia. This release is timed to coincide with host budburst and leaf unfurling (Brook, 1976; MacHardy and Gadoury, 1986; Szkolnik, 1969). Infection risk is greatest early in the growing season when leaves and fruit are young and at their most susceptible (Schwabe, 1979; Schwabe *et al.*, 1984; Xu and Robinson, 2005).

The germ tubes arising from ascospores penetrate through the cuticle, either directly or via an appressorium (Keitt and Jones, 1926; Smereka *et al.*, 1987), and develop into multilayered, pseudoparenchymatous structures, termed stromata, that are presumed to obtain nutrients from the subcuticular space (Fig. 1). The stromata, and the conidia that they produce, cause the characteristic leaf and fruit lesions that give the disease the name of scab or, in some countries, black spot (Fig. 2). Conidia are disseminated by wind and rain from lesions and allow secondary infection to occur within the orchard throughout the fruit development period. These spores, produced asexually, are responsible for an increase in disease if suitable weather conditions occur during the growing season (Fig. 3).

a.

b.

Fig. 1 Formation of stomata during leaf infection by *Venturia inaequalis*: at 5 days post-inoculation, with stomata beginning to form (a), and 10 days post-inoculation, with sporulating stomata (b). Scale bar represents 50 μm . (Reprinted from Bowen *et al.*, 2009. Copyright 2009 with permission from John Wiley & Sons.)

The severe impact of *V. inaequalis* on apple production (MacHardy, 1996) results from the very low level of scab that can render fruit unmarketable. Most commercial apple cultivars are susceptible to scab (Gessler *et al.*, 2006; Holb, 2007). Consequently, a very high level of costly disease control is required to reduce infection in the orchard (Beresford and Manktelow, 1994; Manktelow *et al.*, 1996). Fungicide spray programmes during spring and summer are required in most apple-growing regions for conventional, integrated fruit production and organic production systems (Holb, 2006). *V. inaequalis* has successively developed resistance to dodine, benzimidazole, demethylation inhibitor (DMI; Köller, 1994) and quinone outside inhibitor (Qo;

Fig. 2 Typical scab symptoms on apple fruit caused by *Venturia inaequalis*.

Köller *et al.*, 2004) classes of fungicides. Strategies to delay the development of resistance to these fungicides in field populations of the pathogen rely on restricting the number of applications per season of fungicides in each class and mixing or alternating at-risk fungicides with ones that are not at risk from resistance development (Brent and Holloman, 2007).

The molecular basis for resistance has been identified for these major classes of fungicides, except dodine. Resistance to benomyl (a benzimidazole) in the field is a result of specific mutations in the gene encoding the target protein β -tubulin that modify amino acid 198 (from glutamate to alanine, lysine or glycine; Koenraadt *et al.*, 1992). The mechanism of resistance to the DMI fungicides has not been as well characterized, but appears to involve an energy-dependent efflux system (Palani and Lalithakumari, 1999). Although cross-resistance to these fungicide classes is not observed, it has been noted that resistance to either benomyl or DMI occurs at a higher frequency in *V. inaequalis* isolates already resistant to dodine compared with sensitive isolates (Köller and Wilcox, 2001). The newer QoI class of fungicides target the Qo centre of cytochrome *b*. In the laboratory, resistance can occur either through the upregulation of an alternative oxidase pathway or a G143A mutation in the gene encoding cytochrome *b* itself (Zheng *et al.*, 2000). Upregulation of the alternative oxidase pathway appears to be repressed *in planta*, rendering *V. inaequalis* sensitive; thus these types of mutants are not found in the field. Because cytochrome *b* is encoded by the mitochondrial genome, the G143A mutation must accumulate to a certain level before resistance develops. Thus, resistance development in the field can be delayed by limiting the number of applications of QoI fungicides each season (Köller *et al.*, 2004).

Sanitation practices that reduce ascospore inoculum include autumn urea sprays, which suppress the pathogen's sexual stage

Fig. 3 The life cycle of *Venturia inaequalis*. Subcuticular mycelium = stroma. (This diagram was published in Agrios, *Plant Pathology*, p. 506. Copyright Elsevier 2005.)

and enhance leaf litter breakdown (Burchill *et al.*, 1965), leaf shredding or mulching and the application of lime sulphur or synthetic fungicides in autumn (Holb *et al.*, 2006).

Apple scab is most problematic in regions in which frequent rainfall during spring results in ascospore release and infection. Meteorological criteria defining the duration of wetness required for infection at different temperatures were first proposed by Mills and LaPlante (1954), and are known as Mills' periods. These criteria have become a standard tool, in conjunction with electronic weather monitoring and computer systems, for identifying when conditions suitable for infection occur, so that fungicide applications can be targeted effectively. Other predictive models to help manage fungicide applications against apple scab include ascospore maturation models (MacHardy and Gadoury, 1986) and orchard leaf canopy development models (Beresford *et al.*, 2004).

Although our in-depth understanding of the epidemiology of apple scab has reduced fungicide usage, scab control in most

apple-growing regions is still dependent on the significant input of agrochemicals. This is likely to continue until effective and durable scab resistance is incorporated into new apple cultivars.

THE SUSCEPTIBLE INTERACTION

Nusbaum and Keitt (1938) were the first to report the cytology of the interaction between *V. inaequalis* and apple. They recorded that conidia and ascospores germinate, most commonly from the apical end of the spore, when free moisture is available on the leaf surface. The spore and germ tube adhere to the cuticle, effected by the formation of adhesive mucilage, composed of proteins and carbohydrates (β -galactose and *N*-acetylglucosaminyl residues; Schumacher *et al.*, 2008; Smereka *et al.*, 1988). Usually, a relatively simple appressorium differentiates, but is not essential for penetration (Fig. 4). The appressorium wall is not highly melanized, although a melanin ring is located at the base of the penetration peg (Steiner and Oerke, 2007). Indeed, mechanical

Fig. 4 (a) A spore of *Venturia inaequalis* 9 h post-inoculation, with germination from the apical end of the conidium and formation of an appressorium adhering to the leaf surface. Scale bar represents 5 μm . (b) A germinated conidium and appressorium of *V. inaequalis* adhering to a slide coated in apple wax, 24 h post-inoculation; mr, melanized ring at the base of the penetration peg beneath the appressorium. Scale bar represents 10 μm .

pressure is not required (Smereka *et al.*, 1988), but localized enzymatic hydrolysis of the cuticle is proposed to effect penetration. Extracellular cutinase activity has been implicated, as this enzyme is produced by germinating conidia, and a cutinase inhibitor can prevent penetration (Köller and Parker, 1989; Köller *et al.*, 1991). In addition, esterase-like activity has been reported during the germination of conidia and in appressoria (Nicholson *et al.*, 1972). Interestingly, in infected areas, the cuticle appears to expand significantly, up to 10 times thicker than normal (Kucheryava *et al.*, 2008). Whether this is a result of enzyme action or the secretion of mucilage is not known (Fig. 5).

Once the cuticle is breached, the infection peg differentiates to form subcuticular runner hyphae, which are much wider in diameter than surface mycelia. From these subcuticular hyphae, stromata differentiate at regular intervals. Stromata are made up of laterally dividing cells with diameters of up to 10 μm , markedly different from the tubular hyphae found on the plant surface, agar medium or in liquid culture (Kucheryava *et al.*, 2008). Conidia are formed following enlargement of the uppermost cells of the stroma. *V. inaequalis* appears to have adapted to colonizing the subcuticular apoplastic space during the biotrophic phase of growth. This might explain why the observed induction of callose papillae in the compatible interaction, although implying recognition of the fungus, does not play a role in restricting colonization (Kucheryava *et al.*, 2008). However, it raises the question of why other potential defence mechanisms, such as phytoalexin biosynthesis, appear not to be induced, suggesting that they may be suppressed by effectors that function in the host cytoplasm.

There is no obvious damage to the host throughout stroma development, apart from the breaching of the cuticle. Only on sporulation do the epidermal cells underlying the stroma undergo a progressive depletion of plastids and cytoplasm, accompanied by increasing vacuolation, leading ultimately to necrosis, which is possibly effected by partial cell wall degradation late in the infection cycle (Nusbaum and Keitt, 1938; Valsangiacomo and Gessler, 1988). Cellulase, β -glucosidase, pectinase, and endo- and exo-polygalacturonase activities have been detected in *V. inaequalis* growing *in vitro*. However, if these

Fig. 5 Cross-section of an infected leaf at 14 days post-inoculation, stained with aniline blue, showing the increased thickness of the cuticle (double arrow). Scale bar represents 10 μm ; sh, subcuticular hyphae. (Reprinted from Kucheryava *et al.*, 2008. Copyright 2008 with permission from Elsevier.)

enzymes are only expressed late in infection, when host cell wall degradation is seen, they are unlikely to play a major role in nutrient acquisition during the establishment of infection (Kollar, 1998; MacHardy, 1996; Valsangiacomo and Gessler, 1992). This raises the question as to how the fungus gains nutrients from the host. There is evidence that, like other biotrophic phytopathogens, *V. inaequalis* influences hormone levels at infection sites, specifically cytokinins, to assist with its biotrophic nutrition (Cooper and Ashby, 1998; Walters and McRoberts, 2006). Under this scenario, β -glucosidase activity releases active cytokinin from cytokinin-*O*-glucoside stored in the plant or fungus, in turn creating a cytokinin sink at the site of invasion. Cytokinin accumulation then results in the translocation of nutrients to the fungus at this site (Cooper and Ashby, 1998).

THE RESISTANCE REACTION

Susceptibility is usually associated with unbridled growth of a pathogen on a host. Large spreading lesions caused by *V.*

inaequalis have been observed on fruit and leaves in the field, but whether these originate from one or multiple infection foci has not been established (R. Beresford and K. Plummer, personal observation). Typically, this is not the case, however, with *V. inaequalis* lesions on apple leaves appearing to reach a maximum radius of 1 cm from the point of infection, then ceasing to expand (MacHardy, 1996). This phenomenon has been attributed to ontogenic resistance that develops as leaves and fruit mature, and is complete by the time leaves are fully expanded (Gessler and Stumm, 1984; Schwabe, 1979). Infections become established only on unfolding leaves (Valsangiacomo and Gessler, 1988) and the internodes in between (Jeger and Alston, 1986), with ontogenic resistance developing faster on the ventral than on the dorsal sides of the leaves during their expansion phase (Keitt and Jones, 1926). Ontogenic resistance is slower to develop under glasshouse conditions than in the field, presumably reflecting differential plant tissue maturity rates under different environmental conditions (Szkolnik, 1978). The rates of conidial germination and appressorial differentiation of *V. inaequalis* are not affected by leaf age, but stroma formation and sporulation are reduced in both rate and amount with increasing age of host tissue (Gessler and Stumm, 1984). The basis of this resistance is unknown, but it has been shown that it becomes inactive again late in the growing season when leaves start to senesce and dormant lesions renew their development, even on the oldest leaves (Kollar, 1996). This senescence may compromise the ability of the plant to induce a resistance response. Ontogenic resistance is observable in all apple cultivars and appears never to have been overcome by the pathogen; if the pathogen can build sufficient biomass to enable successful sexual crossing during winter, despite ontogenic resistance operating, it has no selective drivers to overcome this resistance (MacHardy *et al.*, 2001).

Although ontogenic resistance provides protection against scab on mature tissues, growing leaves and fruit remain vulnerable, requiring additional resistance that is predominantly provided by quantitative trait loci (QTLs) and *R* genes. A number of scab resistance QTLs have been identified to date in both glasshouse (Calenge *et al.*, 2004) and field (Liebhard *et al.*, 2003) experiments. Some of these map to the same regions in which major scab *R* genes have been located, e.g. on linkage groups (LGs) 1 and 2 (Calenge *et al.*, 2004; Dunemann and Egerer, 2010; Durel *et al.*, 2003; Soriano *et al.*, 2009). Other QTLs, such as those on LG11 and LG17, are not associated with major genes, with LG17 being a particular 'hot-spot' for broad-spectrum scab resistance QTLs.

QTLs generally confer low levels of partial resistance to a broad spectrum of the pathogen population. In apple, it is increasingly becoming evident, however, that some QTLs are only active against a narrow spectrum of the *V. inaequalis* population. For example, Calenge *et al.* (2004) detected three QTLs

with only a single *V. inaequalis* isolate of the eight that were tested and, although some QTLs had a broad spectrum, it was never complete; none of the 24 QTLs were identified with all isolates tested. An isolate-specific QTL was also identified by Durel *et al.* (2003) in close proximity to the *Rvi6* gene. The individual effects of QTLs are variable, ranging from marked (Calenge *et al.*, 2004) to weak. However, when combined, complementation results in an overall strong resistance. A good example of this is the cultivar 'Antonovka', in which quantitative resistance is the result of a combination of narrow-spectrum genes that together provide a strong and seemingly durable resistance to the pathogen population at large (Gessler *et al.*, 2006).

Despite the durability of ontogenic and QTL-controlled resistance, it is the *R* genes which control the host's participation in, and contribution to, the gene-for-gene relationship between *Malus* and *V. inaequalis* that have attracted the attention of many researchers. This is a result of the simplicity of their genetic analysis and manipulation in relation to breeding material, combined with the relative ineffectiveness of ontogenic resistance for protection of the plant throughout the growing season. To date, 17 *R*-avirulence (*Avr*) gene pairings have been defined (Table 1), the outcome of the interactions of their products determining whether a susceptible or resistant reaction occurs between host and pathogen.

Based on foliar resistance reactions, apple *R* genes can be grouped into three predominant resistance classes exhibiting distinctive resistance responses: the classical hypersensitive response (HR) in which fungal growth is normally terminated very rapidly on penetration, e.g. conditioned by *Rvi4* and *Rvi5* (Win *et al.*, 2003) and *Rvi15* (Galli *et al.*, 2010a); responses involving limited subcuticular growth inducing stellate necrosis, e.g. conditioned by *Rvi2* and *Rvi8* (Bus *et al.*, 2005); and chlorosis, often accompanied by limited sporulation, hence providing only partial resistance, e.g. conditioned by *Rvi6* and *Rvi12* (Fig. 6).

When an HR is involved, the response is often induced immediately after the cuticle is penetrated. For example, in the case of the *Rvi5* gene, the pin-point pit resistance reactions (visible macroscopically as small depressions in the leaf surface) typical of an HR are visible 2–3 days after infection. However, when other *R* genes are involved, e.g. *Rvi15*, it may take 10–11 days for the reaction to be obvious (Galli *et al.*, 2010a; Shay and Hough, 1952). At the epicentre of the response zone, the epidermal cells collapse, creating the small pit. The response goes beyond the initially affected cell into the palisade mesophyll and neighbouring epidermal cells, often affecting a large number of cells, presumably involving cell-to-cell signalling. The response is readily visualized as these cells autofluoresce under ultraviolet (UV) light (Fig. 7). The cells also undergo ultrastructural modifications, such as cell wall apposition and changes in the cellular

<i>Malus</i>				<i>Venturia inaequalis</i>		
Differential host		Resistance locus		Avirulence locus		
No.	Accession	Old	New	New	Old	Race
h(0)	(Royal) Gala		—	—		0
h(1)	Golden Delicious	<i>Vg</i>	<i>Rvi1</i>	<i>AvrRvi1</i>		(1)
h(2)	TSR34T15	<i>Vh2 = Vr - A</i>	<i>Rvi2</i>	<i>AvrRvi2</i>	<i>p-9</i>	(2)
h(3)	(F1 of) Geneva	<i>Vh3</i>	<i>Rvi3</i>	<i>AvrRvi3</i>	<i>p-10</i>	(3)
h(4)	TSR33T239	<i>Vh4 = Vx = Vr1</i>	<i>Rvi4</i>	<i>AvrRvi4</i>		(4)
h(5)	9-AR2T196	<i>Vm</i>	<i>Rvi5</i>	<i>AvrRvi5</i>		(5)
h(6)	Priscilla	<i>Vf</i>	<i>Rvi6</i>	<i>AvrRvi6</i>		(6)
h(7)	(F1 of) <i>M. × floribunda</i> 821	<i>Vfh</i>	<i>Rvi7</i>	<i>AvrRvi7</i>		(7)
h(8)	GMAL3631-W193B	<i>Vh8</i>	<i>Rvi8</i>	<i>AvrRvi8</i>		(8)
h(9)	(F1 of) Dolgo	<i>Vdolgo</i>	<i>Rvi9</i>	<i>AvrRvi9</i>	<i>p-8</i>	(9)
h(10)	A723-6	<i>Va</i>	<i>Rvi10</i>	<i>AvrRvi10</i>		(10)
h(11)	A722-7	<i>Vbj</i>	<i>Rvi11</i>	<i>AvrRvi11</i>		(11)
h(12)	Hansen's baccata #2	<i>Vb</i>	<i>Rvi12</i>	<i>AvrRvi12</i>		(12)
h(13)	Durello di Forli	<i>Vd</i>	<i>Rvi13</i>	<i>AvrRvi13</i>		(13)
h(14)	(F1 of) Dölmener Rosen	<i>Vdr1</i>	<i>Rvi14</i>	<i>AvrRvi14</i>		(14)
h(15)	GMAL2473	<i>Vr2</i>	<i>Rvi15</i>	<i>AvrRvi15</i>		(15)
h(16)	MIS op 93.051 G07-098	<i>Vmis</i>	<i>Rvi16</i>	<i>AvrRvi16</i>		(16)
h(17)	(F1 of) Antonovka APF22	<i>Va1</i>	<i>Rvi17</i>	<i>AvrRvi17</i>		(17)

Table 1 Nomenclature of the differential host–pathogen interactions of *Venturia inaequalis* and *Malus*. Research is in progress to identify F1 progeny in current differential hosts that carry multiple resistance genes.

Fig. 6 Characteristic scab resistance reactions on apple leaves: pin-point pit, hypersensitive response conditioned by the *Rvi4* gene (left); stellate necrosis by the *Rvi2* gene (middle); and chlorosis with limited sporulation conditioned by the *Rvi6* gene (right) under glasshouse conditions.

organelles; the extent of the ultrastructural changes of the epidermal and palisade mesophyll cells varies with the *R* gene involved (Chevalier and Lespinasse, 1991; Chevalier *et al.*, 1991). On resistant hosts that exhibit stellate necrosis, the fungus grows very fine mycelial structures that often show autofluorescence under UV light (Bus *et al.*, 2005, 2010). The chlorotic resistance reaction shows less autofluorescence under UV light compared with HR and stellate necrosis reactions, and encompasses a wide range of intensity of response.

These differences in phenotypic expression of resistance (HR, stellate necrosis and chlorosis) may reflect differences in the defence signalling cascades and resistance reactions induced as a consequence of different *R* gene-mediated recognition events. In addition, they may also reflect the virulence role of their

cognate effectors, perhaps involving suppression of components of the resistance reaction, or differences in the temporal expression of these effectors.

In the past, some interactions have been classified as compatible as a result of the production of spores by the pathogen. However, in some cases, this sporulation is still preceded by a resistance reaction that is unable to contain the infection, but does slow down pathogen spread and sporulation rate. This was demonstrated with two putative race (4) isolates of *V. inaequalis*, where the resistance conditioned by *Rvi4* is commonly expressed as immunity but, in the case of isolates 1638 and US4, this was reduced to partial resistance (Bus, 2006). Such isolate-dependent variation could indicate the presence of an additional effector in these isolates, able to suppress the defence reaction.

Fig. 7 Microscopic interference blue autofluorescence observations, demonstrating the hypersensitive response involving the epidermis and the palisade mesophyll conditioned by the *Rvi5* gene. Several hypersensitive responses are shown, each initiated by a *Venturia inaequalis* spore (sp) at its centre. Scale bar represents 200 μ m.

MOLECULAR DISSECTION OF *R* GENES

Despite the knowledge of their existence for over half a century, and extensive classical genetic studies on many of the currently identified *R* genes (reviewed by Gessler *et al.*, 2006; MacHardy, 1996), molecular analysis of only one locus has resulted in the cloning and demonstration of the proof of function of an *R* gene active against *V. inaequalis*. The *Rvi6* locus derived from the crabapple species *Malus floribunda* 821 was dissected independently by two groups (Vinatzer *et al.*, 2001; Xu and Korban, 2002), attracting significant interest as it is widely deployed in commercial cultivars (Janick *et al.*, 1996; Korban, 1998). The map-based cloning of the locus was facilitated by the development of a comprehensive array of molecular markers throughout the 1990s (reviewed by Tartarini and Sansavini, 2004). Both groups identified four *R* gene paralogues at the locus (termed *HcrVf1–4* and *Vfa1–4*, respectively; Vinatzer *et al.*, 2001; Xu and Korban, 2002), one of which (*HcrVf3*) appeared to be a truncated pseudogene. The *HcrVf* genes exhibit high sequence similarity to a class of *R* genes found in tomato, that are characterized by multiple N-terminal leucine-rich repeats (LRRs) and transmembrane domains (Dangl and Jones, 2001). They are predicted to encode extracytoplasmic glycoproteins, anchored to the cell membrane, with the LRRs comprising the majority of the extracytoplasmic portion (Vinatzer *et al.*, 2001). The three putative functional genes at the *Rvi6* locus are expressed constitutively, although differentially. *HcrVf1* and *HcrVf2* are highly expressed in immature leaves, but negligibly in mature leaves and, conversely, the expression of *HcrVf4* is higher in mature than in immature leaves.

Early indications that *HcrVf2* is an active *R* gene (Barbieri *et al.*, 2003) were corroborated by Belfanti *et al.* (2004). Belfanti

et al. (2004) transformed susceptible 'Gala' with *HcrVf2*, utilizing the strong constitutive *Cauliflower mosaic virus* (CaMV) 35S promoter. The transgenic lines were only resistant to isolates of *V. inaequalis* that expressed the cognate *AvrRvi6* effector gene. In contrast, when native promoters were used to control either *HcrVf1* or *HcrVf2* expression in susceptible cultivars 'Gala' and 'McIntosh', both genes conferred resistance, although the levels of resistance were lower than those found in conventionally bred cultivars carrying the *Rvi6* gene (Malnoy *et al.*, 2008). This was attributed to a dose effect equivalent to plants homozygous for *Rvi6* having a higher resistance response compared with plants heterozygous for the gene (Gessler *et al.*, 1997; Tartarini *et al.*, 2000), or a requirement for both *HcrVf1* and *HcrVf2* for effective resistance. However, subsequent optimization of the native promoter length resulted in resistance equivalent to that exhibited by cultivars with *Rvi6* introgressed via classical breeding being achieved with *HcrVf2* in a 'Gala' or 'Elstar' background (Silfverberg-Dilworth *et al.*, 2005; Szankowski *et al.*, 2009). LRR receptor-like protein kinases may also be involved in pathogen perception, downstream of the *Rvi6* gene product, as the expression of two genes belonging to the *LRPKm* family is upregulated during a resistance response in the presence of *HcrVf2* (Cova *et al.*, 2010; Komjanc *et al.*, 1999).

Although *R* genes have been cloned, many questions still remain as to how they and related genes operate; many genes homologous to *HcrVf* genes have been found in resistant hosts in addition to *M. floribunda* 821 (Boudichevskaia *et al.*, 2009) and its derivatives (Broggini *et al.*, 2009b). The *HcrVf2* gene, and its homologues at the *Rvi6* locus, is most closely related to the *Cf* genes of tomato for resistance to *Cladosporium fulvum*, particularly those belonging to the *Hcr9* (homologues of *C. fulvum* resistance gene *Cf-9*) gene family (*Cf-4*, *Cf-4E*, *Cf-9B*, *Cf-9*; Jones *et al.*, 1994; Kruijt *et al.*, 2005; Laugé *et al.*, 1998; Parniske *et al.*, 1997; Takken *et al.*, 1998; Thomas *et al.*, 1997). This similarity in *R* gene may reflect the similarity in pathogenic strategy adopted by the two pathogens and the effectors produced; they both colonize the plant extracellularly, occupying either the leaf apoplast, in the case of *C. fulvum*, or the subcuticular space, in the case of *V. inaequalis*. The proteins predicted to be encoded by *HcrVf* and the *Hcr9* gene family show the same overall structure with similar domains, with the same domains exhibiting the most variability [the cysteine-rich B domain and the N-terminus of the LRR domain (C); Kruijt *et al.* 2005]. The *HcrVf* and *Hcr9* gene clusters share the same structure with tandemly repeated genes residing at a complex locus. They may also share a similar mode of evolution involving duplication events and positive selection for diversification in the highly variable domains (Kruijt *et al.*, 2005; Xu and Korban, 2004). However, inferences as to whether the *HcrVf* gene products operate by a direct interaction with their cognate effector or are a component of a guard system are harder to draw, as members of the *Hcr9* gene family may

operate by either method. In the Cf-9-Avr9 system, there is a high-affinity Avr9-binding site that suggests that this may be a guarded system (Kooman-Gersmann *et al.*, 1996). Similarly, Cf-9B may also be a 'guarding' *R* gene as its cognate effector interacts with a necrosis-inducing factor (Chakrabarti *et al.*, 2009). In contrast, Cf-4 is thought to interact directly with Avr4, but no binding experiments have demonstrated this categorically (van Esse *et al.*, 2007; Wulff *et al.*, 2009).

Recently, three clustered genes were identified as candidates for the *R* gene *Rvi15* in accession GMAL 2473. All three genes were found to be transcribed in young leaf tissue and to share a similar structure: an N-terminus homologous to the Toll and mammalian interleukin-1 receptor proteins (TIR), a central nucleotide-binding site (NBS) and an LRR domain at the C-terminus (Galli *et al.*, 2010b). Whether these genes are responsible for the *Rvi15* resistance phenotype remains to be established. However, if the *Rvi15* status is confirmed, this too raises questions regarding *V. inaequalis* effector biology and the recognition events leading to resistance. TIR-NBS-LRR (TNL) *R* proteins were originally thought to be localized exclusively to the cytoplasm, but have been shown recently to be found in a variety of intracellular compartments, often associated with membrane systems, with their putative recognition domains exposed to the cytoplasm (Rafiqi *et al.*, 2009). Galli *et al.* (2010b) postulated that the *Rvi15* protein may recognize cuticle or cell wall degradation products that pass into the cytoplasm, resulting from the subcuticular action of an effector from *V. inaequalis*. An alternative hypothesis is that *V. inaequalis* targets an effector to the host cytoplasm, where it exerts an effect as a suppressor of host defence, enabling *V. inaequalis* to remain subcuticularly without eliciting an effective resistance response. There are precedents for filamentous fungal effectors to be taken up intracellularly and to act as suppressors of host defence (Houterman *et al.*, 2008, 2009); hence *V. inaequalis* may not merely be secreting effectors to the subcuticular space as previously thought. This supposition is supported by the downregulation of the expression of two LRR receptor-like protein kinase genes following inoculation with *V. inaequalis*. These genes are putatively involved in plant basal defence, and their downregulation may be a result of the action of a suppressor from *V. inaequalis* (Cova *et al.*, 2010).

Insights into the *C. fulvum*–tomato interaction may shed light on the nature of ontogenic resistance in *Malus*. Cf-9B confers resistance to mature plants of tomato against *C. fulvum*, but not seedlings, although it is expressed in young plants (Panter *et al.*, 2002). This suggests that the cognate effector of Cf-9B, Avr9B, may target a protein that is guarded by Cf-9B, the expression of which is only upregulated in mature plants (Wulff *et al.*, 2009). A similar system could be operating to confer, or contribute to, ontogenic resistance in *Malus*, albeit not mediated via an HR, which, because of its ubiquitous nature, has not been genetically

dissected. *HcrVf4* at the *Rvi6* locus is highly expressed in mature leaves, although it is still expressed in immature leaves (Xu and Korban, 2002). Such a gene could be guarding a host target that is expressed at increasing levels throughout maturation, a target for an effector from *V. inaequalis*.

WGS for apple (cv. 'Golden Delicious') is now available [Istituto Agrario di San Michele all'Adige (IASMA), unpublished results]. As other *Malus* spp. also become sequenced, additional *R* genes will become available. However, it is not possible to ascertain *a priori* which *R* genes recognize particular pathogens or to predict their durability from sequence alone. The durability of an *R* gene is a function of its cognate effector. *R* genes that match effectors that are redundant or dispensable are unlikely to be durable, whereas *R* genes that recognize essential effectors are likely to be so. Therefore, the key to durable resistance is the pyramiding of *R* genes that recognize effectors with significant roles in pathogenicity. The identification and functional characterization of the effector complement from *V. inaequalis* are crucial for this goal.

MOLECULAR APPROACHES FOR THE IDENTIFICATION OF EFFECTOR GENES OF *V. INAEQUALIS*

Several molecular approaches have been employed to identify and clone effector genes of *V. inaequalis*. These include the forward genetic techniques of expressed sequence tag (EST) database mining, as well as map-based cloning (Broggini *et al.*, 2007) and a reverse genetics proteomics approach (Gau *et al.*, 2004; Win, 2003; Win *et al.*, 2003).

EST database mining

The first large-scale analysis of ESTs derived from the *V. inaequalis*–*Malus* pathosystem resulted in the identification of 4215 ESTs (clustered into 2090 nonredundant sequences) from five distinct cDNA libraries (Bowen *et al.*, 2009). Two of the five were *in planta* libraries: one prepared from infected leaf material of a compatible interaction between *V. inaequalis* and apple, and the other prepared by probing the former with genomic DNA of *V. inaequalis* to enrich for fungal sequences (Newcomb *et al.*, 2006). The remaining three were *in vitro* libraries: two generated from mycelia grown in potato dextrose broth shake cultures, and one, a suppression subtractive hybridization library, prepared from growth on cellophane overlying potato dextrose agar, subtracted against growth on potato dextrose agar alone, to enrich for cellophane-induced genes (Bowen *et al.*, 2009). Combined, a total of 937 ESTs were classified as putatively fungal in origin, and could be assembled into 633 nonredundant sequences (Bowen *et al.*, 2009). Mining of this EST database resulted in the identification of 16 candidate effector genes (*Vice1*–*Vice16*; *Ven*–

turia inaequalis candidate effector) based on features common to the characterized effector genes from filamentous fungi, i.e. they encode small (<400 amino acids in length), novel, cysteine-rich proteins, with a putative signal peptide (Rep, 2005; Stergiopoulos and de Wit, 2009; Templeton *et al.*, 1994; de Wit *et al.*, 2009), although candidates were not required to meet all of these criteria. Three of the 16 candidates, specifically *Vice4*, *Vice12* and *Vice16*, were shown to be significantly upregulated during growth *in planta* compared with liquid growth *in vitro* (Bowen *et al.*, 2009).

Growth of *V. inaequalis* within cellophane can be viewed as a potential *in vitro* model for subcuticular growth and morphogenetic differentiation *in planta* (Kucheryava *et al.*, 2008). This is corroborated by the expression of two cellophane-induced genes, *cin1* and *cin3*, which are upregulated over 1000-fold *in planta*, compared with liquid growth *in vitro* (Kucheryava *et al.*, 2008). For *cin1*, mRNA expression is strongly upregulated during the early stages of infection [5 days post-inoculation (dpi)], whereas, for *cin3*, expression is strongly upregulated at both 5 and 10 dpi. Both genes are novel, and encode secreted proteins with an imperfect repeat domain structure, in which the repeat number and diversity vary in an isolate-specific manner (Kucheryava *et al.*, 2008). The repetitive nature of Cin1 and Cin3 is of particular interest, given the role played by some secreted phytopathogenic fungal proteins with tandem repeats in the pathogenesis of a host plant (Müller *et al.*, 2008). To simplify future structural determination of the Cin1 protein using nuclear magnetic resonance spectroscopy, a two-domain truncated version has been purified to homogeneity (Jones *et al.*, 2009). Functional analysis of the *Vice* candidates, *cin1* and *cin3*, is currently being performed using RNA interference (RNAi).

RNAi has been used to silence the trihydroxynaphthalene reductase (*THN*) gene involved in the melanin biosynthesis pathway (Fitzgerald *et al.*, 2004). Melanin is required for the pathogenesis of several phytopathogenic fungi that penetrate their host through the production of a highly melanized appressorium (Henson *et al.*, 1999). Under *in vitro* conditions, *THN*-silenced transformants of *V. inaequalis* exhibited a distinctively modified light-brown phenotype, but retained their ability to infect apple, although the degree of infection was not quantified (Fitzgerald *et al.*, 2004).

Comprehensive EST collections have also been produced from the host plant (*M. × domestica*, *M. baccata* and *M. sieboldii*). These are derived from several tissues, stress treatments and genotypes (Gasic *et al.*, 2009; Newcomb *et al.*, 2006), and include a subset of *V. inaequalis*-modulated apple genes (Paris *et al.*, 2009). Some of the tissues for these libraries were collected from orchard-grown trees; therefore, it is likely that they contain genes corresponding to pathogen sequences as a consequence of pathogen contamination of host material. Taken together, these EST libraries provide a valuable genomic resource

for *V. inaequalis* and *Malus* spp., particularly *M. × domestica*, and provide important information for understanding the molecular basis of the *V. inaequalis*–*Malus* interaction.

Map-based cloning

Maps generated from phenotypic markers have been expanded upon recently by the incorporation of molecular markers: amplified fragment length polymorphisms and SSRs (Guérin *et al.*, 2004). These maps are now up to 950 and 1053 cM in length and span 11–14 LGs (Xu *et al.*, 2009; G. A. L. Brogini and V. G. M. Bus, unpublished results). In contrast, seven chromosomes were identified by cytology (Day *et al.*, 1956), although other earlier cytological estimates range from four to eight (reviewed in Boone, 1971). Accurate determination of chromosome number will be supported by the resolution of the complete WGS, whole chromosome optical maps of *V. inaequalis* and the physical resolution of chromosomes by pulsed-field gel electrophoresis.

Despite the ease of accomplishing *in vitro* mating in *V. inaequalis*, a map-based approach for cloning effector genes has not yet borne fruit. Combining sequence characterized amplified region and SSR markers with a bacterial artificial chromosome library enabled Brogini *et al.* (2007) to isolate a 330-kb region that encompasses the *AvrRvi1* locus. A bioinformatics search of this contiguous sequence resulted in the identification of 40 open reading frames coding for proteins that were predicted to be secreted via either a signal peptide or a nonclassical secretion pathway (Brogini *et al.*, 2009a). Expression and functional analyses of these candidates are ongoing.

Proteomics

A proteomics approach has proven to be effective for the identification and cloning of effector genes from phytopathogenic fungi. For example, all the effector genes of *C. fulvum* and *Fusarium oxysporum* f. sp. *lycopersici* identified to date have been cloned using a reverse genetics proteomics approach (van der Does *et al.*, 2008; van Esse *et al.*, 2007, 2008; Houterman *et al.*, 2007; van Kan *et al.*, 1991). In these studies, effector proteins were characterized from fluids extracted from infected host tissues. A similar approach was conducted for scab-infected apple leaves, but no fungal proteins were identified, although the regulation of some pathogenesis-related proteins in response to scab infection was elucidated (Gau *et al.*, 2004).

In an early study, Raa and Sijpesteijn (1968) reported 'toxins' in liquid cultures of *V. inaequalis* capable of eliciting rapid leaf wilt in resistant, but not susceptible, seedlings of apple derived from an 'Antonovka 34-20' (resistant) and 'Golden Delicious' (susceptible) cross. These 'toxins' are potentially effector proteins, with the leaf wilt reminiscent of a rapid, severe HR. The differential action of these putative effectors was abolished by

Table 2 Effector genes from filamentous fungal pathogens with orthologues in the *Venturia inaequalis* whole genome sequence (WGS).

Gene	Similarity score against <i>Venturia inaequalis</i> WGS	Organism	Host	Biological activity	Involvement in virulence/pathogenicity	Corresponding <i>R</i> gene	No. cysteine residues
<i>Ecp6</i>	6e-43	<i>Cladosporium fulvum</i>	Tomato	LysM domains: chitin binding to scavenge chitin to prevent recognition by plant	Required for full virulence		8
<i>AvrLm6</i>	3e-08	<i>Leptosphaeria maculans</i>	Oil seed rape	Induces an HR	Not required for full virulence	<i>Rlm6</i>	6
<i>Avr-Pita</i>	3e-11	<i>Magnaporthe oryzae</i>	Rice	Putative zinc metalloprotease	Not required for pathogenicity	<i>Pi-ta</i>	8

HR, hypersensitive reaction.

boiling the culture filtrate and, following gel filtration-mediated fractionation of the culture filtrate, paper chromatography yielded spots positive for ninhydrin, suggesting a proteinaceous nature. Unfortunately, the putative effectors and the cognate host *R* genes used in this study were not identified (Raa and Sijpesteijn, 1968).

This approach was pursued further with the aim of cloning the *AvrRvi5* effector gene of *V. inaequalis* (Win *et al.*, 2003). Compounds secreted by *V. inaequalis* into liquid culture were investigated for their ability to elicit cell death, reminiscent of an HR, in a host plant carrying the cognate *Rvi5* *R* gene. At least three major and three minor candidates for the *AvrRvi5* effector protein were identified in the purest fraction of the cell death-inducing cell-free culture filtrate. This fraction also elicited cell death in a host plant carrying *Rvi4*, suggesting that it may also contain *AvrRvi4*, or an effector with dual host specificities to both *R* genes. Full-length cDNA transcripts for three of the *AvrRvi4,5* candidate proteins were then identified from EST libraries. Sequence similarity was found to an allergen protein, a protein involved in cell adhesion and a β -1,3-endoglucanase enzyme (Win, 2003). The allergen-like protein and putative β -1,3-endoglucanase were also identified as major components of proteins isolated from scab-infected leaves of a compatible interaction (Fitzgerald, 2004). In this study, proteins were isolated from *V. inaequalis* race (1)-infected apple seedling leaves (grown from seeds of open-pollinated apple cv. 'Royal Gala'). Various methods, similar to those used for the isolation of proteins from tomato-*C. fulvum* interactions (van Kan *et al.*, 1991), including the extraction of total proteins and the assay of extracts from vacuum-infiltrated leaves, were trialled, but were unsuccessful for the isolation of fungal proteins from infected apple leaves. The subcuticular location of *V. inaequalis* led to the adoption of a different approach, in which the surface of infected leaves was rubbed gently with carborundum grit to disrupt the cuticle and release the fungal proteins. Using this method, novel fungal proteins were identified and are under further investigation. In addition, nucleotide sequences of the allergen-like genes from avirulent and virulent isolates were identical, suggesting that the *bona fide* effectors of the cell death-inducing fraction were not

represented in the EST libraries, although these candidates have yet to be ruled out.

THE WGS AND FUTURE DIRECTIONS

The identification of genes coding for effectors from *V. inaequalis* has been challenging. Reverse genetics and proteomics approaches have been hampered by the low volume of apoplastic fluid that can be extracted from infected apple leaves. ESTs have been useful, but are limited by the low percentage of transcripts that can be confirmed as being of fungal origin in infected leaves. Both of these approaches will be advanced by the application of new sequencing technologies, which will provide WGS and transcriptomes with deeper sequence penetration. WGS will enable the identification of effectors by similarity to known effectors from related fungi and by selecting for small secreted novel proteins, especially those that are cysteine rich. Furthermore, WGS will greatly facilitate the identification of proteins using proteomics.

Three laboratories, including a consortium led by Genoscope (<http://www.genoscope.cns.fr/spip/>), are currently generating *V. inaequalis* WGS. It is likely that a complete genome will be available in 2010. Preliminary data from our laboratory suggest that there are approximately 36 Mb of nonrepeated DNA. Approximately 95% of genes can be found in these sequences, as 94 candidate effectors, house-keeping genes and other ESTs characterized in previous work were found to have strong BLAST hits to the draft WGS, as did 33 of 37 SSR markers. Orthologues of several fungal effector genes were identified, including *Ecp6*, *AvrLm6* and *Avr-Pita* (Bolton *et al.*, 2008; Fudal *et al.*, 2007; Orbach *et al.*, 2000; Table 2). Moreover, amino acid sequence information from a candidate *AvrRvi4,5* effector, not present in EST libraries, that elicited a cell death response on hosts 4 and 5 (Win, 2003), exactly matched part of a novel 154-amino-acid protein, with a signal peptide and eight cysteines, that was predicted from the WGS. Functional analysis of the corresponding gene is currently underway by gene silencing. Functional analysis utilizing a disruption strategy, requiring ready access to flanking DNA, will now be more easily attempted with the

increased availability of sequence data, although the feasibility of gene disruption has yet to be tested in *V. inaequalis*. Further sequencing, including transcriptome analysis and paired-end reads, will be carried out to reduce the number of scaffolds in the assembly and to aid annotation.

Many Dothideomycete genomes are currently being sequenced, mainly by the Broad Institute (<http://www.broadinstitute.org/>), the Joint Genome Institute (<http://www.jgi.doe.gov/>) and Genoscope. A few genomes have been completed (e.g. *Mycosphaerella graminicola*, *Mycosphaerella fijiensis*, *Stagonospora nodorum* and *Pyrenophora tritici-repentis*) and many are nearing completion (e.g. *Cochliobolus heterostrophus*, *Dothistroma septosporum*, *Leptosphaeria maculans*). Plant pathogens are well represented in the Dothideomycetes, and thus comparative genomics may well prove to be invaluable in the identification of effectors in this fungal class. A low rate of sequence conservation of filamentous fungal effectors has, until now, hampered their discovery. Although this may be attributable to a high rate of evolution of effector genes, it may merely be a result of the, so far, sparse genome coverage of many closely related plant pathogens. Recently, orthologues of *C. fulvum* effector genes *Avr4* and *Ecp6* have been found in many fungal species (Bolton *et al.*, 2008); indeed, there are functional orthologues of *Avr4* and *Ecp2* in *M. fijiensis* and their encoded effectors are able to interact with the respective tomato *R* gene products (Stergiopoulos *et al.*, 2010), and a functional homologue of the *ToxA* peptide gene from *S. nodorum* was found in the *P. tritici-repentis* genome (Friesen *et al.*, 2006, 2008). This trend is repeated in the *V. inaequalis* WGS, with the presence of orthologues of *Ecp6*, *AvrLm6* and *Avr-Pita*. This conservation of effector sequence may be an indication of the effectiveness of function of the orthologues across a wide host range, or a measure of the essential nature of the effectors with the fitness cost of loss or mutation too great. If the effectors are essential, their cognate *R* genes may well be durable; thus, with the advent of comparative genomics using the WGS of *V. inaequalis*, the identification of key effectors and durable cognate *R* genes is arguably a step closer.

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