GENOMIC ANALYSIS OF *PYRENOPHORA TERES*: AVIRULENCE GENE MAPPING, KARYOTYPING AND GENETIC MAP CONSTRUCTION

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Aaron D. Beattie

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Abstract

Pyrenophora teres Drechs. (anamorph: *Drechslera teres* (Sacc.) Shoem.) is the causal agent of barley net blotch. Net blotch is an economically important disease commonly found throughout the barley producing regions of the world. Significant financial losses result from yield reductions, ranging from 15-35%, and decreased grain quality. Despite its prevalence, it is unclear if the *P. teres*-barley pathosystem follows a gene-for-gene model, and more generally, little is known about its genetic organization. Three studies were initiated to address these questions.

The first study investigated the genetic control of avirulence in *P. teres*. To establish an appropriate study system, a collection of ten net form (*P. teres* f. *teres*) and spot form (*P. teres* f. *maculata*) isolates were evaluated on a set of eight differential barley lines to identify two isolates with differential virulence on a specific host line. WRS 1906, exhibiting low virulence on the cultivar 'Heartland,' and WRS 1607, exhibiting high virulence, were mated and 67 progeny were isolated and phenotyped for virulence on Heartland. The population segregated in a 1:1 ratio, 34 avirulent to 33 virulent ($\chi^2 = 0.0$, $P = 1.0$), indicating single gene control of WRS 1906 avirulence on Heartland. Bulked segregant analysis was used to identify six amplified fragment length polymorphism (AFLP) markers closely linked to the avirulence gene ($Avr_{Heartland}$). This work provides evidence that the *P. teres*-barley pathosystem conforms to the gene-for-gene model.

In the second study, five isolates of *P. teres*, representing both net and spot forms, were analyzed by the germ tube burst method (GTBM) and pulsed field gel electrophoresis (PFGE) to determine the species' karyotype. Nine chromosomes were observed in all isolates using the GTBM and estimation of chromosome lengths varied from 0.5 to 3.0 μm. PFGE separated 7 to 8 bands depending on isolate, but analysis of bands by densitometry indicated nine chromosomes. Chromosome size ranged from 1.8 to ~6.0 Mb providing a genome size estimate of 32 to 39 Mb. Significant chromosome-length polymorphisms (CLP) were observed between isolates. These CLP did not hinder

mating between mating-type compatible net form isolates. No particular CLP or individual chromosome could be associated with differences in disease symptoms observed between pathogen forms. This study provides the first karyotype of both *P. teres* forms and will assist genetic mapping of this pathogen.

A genetic linkage map of *P. teres* f. *teres*, was constructed in the third study using the population of 67 progeny derived from the WRS 1906 × WRS 1607 cross. The map consists of 138 markers including 114 AFLPs, 21 telomere RFLPs, the mating-type (*MAT*) locus and an avirulence locus ($Avr_{Heartland}$) controlling interaction with barley cultivar 'Heartland.' Markers were distributed across 24 linkage groups ranging in length from 2 to 110 cM with an average marker interval of 8.5 cM. The total map length was 797 cM. A telomere-specific probe, consisting of the sequence $(TTAGGC)_4$, was used to map 15 of the 18 telomeres. One of these telomeres mapped to within 3 cM of the $Avr_{Heartland}$ locus. Attempts to consolidate linkage groups by hybridizing markers to the electrophoretically separated chromosomes was unsuccessful because probes bound to multiple chromosomes, likely due to repetitive DNA within the probe. This is the first genetic map reported for this species and it will be a useful genetic tool for map-based cloning of the $Avr_{Heartland}$ gene tagged in this study.

This research has provided a number of new insights into the net blotch pathogen and provides a useful research tool in the form of a genetic map. This information lays the foundation for further genetic study of *P. teres* and will complement studies on barley resistance to net blotch that may potentially lead to more durable resistance.

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List of Abbreviations

AD: acidic transcriptional activation domain

AFLP: amplified fragment length polymorphism

Avr: avirulence gene

Avr: avirulence protein

BSA: bulked segregant analysis

C-terminal: carboxy end of a protein

CC: coiled-coil

CLP: chromosome-length polymorphism

DH: doubled haploid

effector: synonym for avirulence protein

ET: ethylene

flg22: 22 amino acid domain of bacterial flagellin

FLS2: flagellin insensitive 2

GTBM: germ tube burst method

HR: hypersensitive response

hrc: hypersensitive response and conserved

hrp: hypersensitive response and pathogenicity

HST: host-selective toxin

JA: jasmonic acid

LeEix: *Lycopersicon esculentum* ethylene-inducing xylanase

LG: linkage group

LPS: lipopolysaccharides

LRR: leucine-rich repeat

MAPK: mitogen-activated protein kinase

N-terminal: amino end of a protein

NBS: nucleotide binding site

NDR1: non-race-specific disease resistance 1

NLS: nuclear localization signal

NPP1: necrosis-inducing *Phytophthora* protein 1

PAD4: phytoalexin deficient 4

PAMP: pathogen-associated molecular pattern

PBS1: AvrPphB susceptible 1

PCD: programmed cell death

PFGE: pulsed field gel electrophoresis

PR: pathogenesis-related

Prf: *Pseudomonas* resistance and fenthion sensitivity

Pti: Pto interacting

Pto: *Pseudomonas syringae* pv. *tomato*

QTL: quantitative trait loci

R: resistance gene

R: resistance protein

RIN4: RPM1 interacting 4

RLK: receptor-like kinase

RLP: receptor-like protein

ROS: reactive oxygen species

RPM1: resistance to *Pseudomonas syringae* pv. *maculicola* 1

RPP: resistance to *Peronospora parasitica*

RPS: resistance to *Pseudomonas syringae* pv. *tomato*

SA: salicylic acid

SAR: systemic acquired resistance

SOD: superoxide dismutase

TIR: Toll and interleukin-1 receptor

TTSS: type III secretion system

WRKY: tryptophan(W)-arginine(R)-lysine(K)-tyrosine(Y)

General Introduction

During the past decade there has been a tremendous increase in the understanding of plant disease resistance. Key concepts such as the resistance (*R*) gene guard hypothesis, *R* gene classes and evolution, avirulence (*Avr*) genes as virulence factors, basal defense and signalling pathway crosstalk have emerged.

The current model of a plant's immune system describes a "non-host" defense network consisting of passive and induced (basal) defenses essential for, and highly effective at, repelling the majority of potentially pathogenic organisms. Recognition of these organisms is accomplished by detecting a variety of structural and secreted compounds commonly found in pathogens such as flagellin, lipopolysaccharides and chitin, collectively referred to as pathogen associated molecular patterns (PAMPs). Plant receptors which interact with these compounds can initiate mitogen-activated protein kinase (MAPK) and salicylic acid (SA) signalling pathways leading to a number of defense reactions such as pathogenesis-related (PR) proteins, cell wall thickening and papillae.

Despite the effectiveness of the non-host defense system, over the course of evolution some organisms have managed to circumvent it to become pathogenic species. The various means by which this is accomplished has provided an explanation for the presence of avirulence genes in pathogens. Long a paradox, it has become increasingly clear that the function of these genes is to assist a pathogen's access to a host plant. Avirulence genes do this by subverting key regulatory proteins involved with basal defense or the better known hypersensitive response. In response to these pathogens, plants have developed specialized defenses, known as resistance genes, which are layered on top of the basal defense machinery. Indeed, *R* gene products and basal defense receptors access some of the same downstream signalling pathways, perhaps not surprising since they share many structural similarities. The fundamental difference between the two systems is the speed and intensity of the response, with *R* gene-

mediated defense being a fast, intense reaction ultimately leading to the hypersensitive response.

The *R-Avr* area is a convenient point from which one can branch out to understand other aspects of the plant-pathogen interaction. To provide context as to when *R* gene defense is activated one must understand the basal and pre-formed defenses that have been bypassed by the pathogen. Once *R* gene defense has been initiated, the pathways activated encompass a wide and complex array of cellular reactions and interactions fundamental to plant defense.

The understanding of *R-Avr* function has resulted from work carried out primarily with *Arabidopsis thaliana* and its associated bacterial pathogens. This has provided a framework for understanding plant-pathogen interactions that will likely be consistent in other pathosystems, but the ubiquity of the details in these concepts remains to be confirmed. As an example, a number of *R* genes cloned from other plant species do not fall within the two major classes noted for *Arabidopsis*. There may also be differences between bacterial and fungal pathogens and, at present, there are a disproportionately large number of *Avr* genes cloned from bacterial pathogens. This is in part due to the larger genome size of fungal and oomycete pathogens and increased difficulty in culturing the obligate biotrophic species. However, these pathogens are responsible for the majority of plant diseases and thus warrant increased study.

This thesis describes a molecular study of the barley net blotch pathogen, *Pyrenophora teres* Drechs. (anamorph: *Dreschlera teres* (Sacc.) Shoem.), initiated to understand the genetic control of avirulence in this fungal pathogen and to address the lack of genetic information available for the pathogen. Three goals were established:

1) to determine if avirulence in *P. teres* is controlled by a single gene and if so, identify markers linked to this gene,
2) to karyotype *P. teres* by pulsed field gel electrophoresis (PFGE) and germ tube burst method (GTBM) and,

3) to create a genetic linkage map of *P. teres*.

These goals will:

1) lay the framework for the cloning of an avirulence gene and contribution to understanding fungal avirulence genes,
2) allow a better understanding of the genomic organization of *P. teres* and,
3) provide a useful research tool in the form of a molecular genetic map that will facilitate the cloning of genes and provide a common reference for researchers working with this organism.

This pathogen was chosen for study for several reasons. First, it is a common, persistent fungal pathogen throughout the barley growing regions of the world and is of economic concern to the barley industry. Second, information obtained will contribute to a better understanding of fungal pathogens. Third, the pathogen possesses a heterothallic, self-sterile mating system making it ideal for producing a segregating mapping population. Finally, a number of studies have shown that a large number of *P. teres* pathotypes exist and that resistance loci can be found throughout the barley genome, suggesting that the barley-net blotch pathosystem may exhibit a classic gene-for-gene interaction controlled by defined pairs of *R* and *Avr* genes.

Discussion of the net blotch pathogen, the barley host as it relates to net blotch resistance, *R* genes, *Avr* genes and their interaction within the larger context of plant defense are presented in the literature review. Three subsequent chapters address the goals stated above.

Chapter 1: Literature Review

The Pathogen: *Pyrenophora teres*

Taxonomy

Pyrenophora teres Drechs. is a filamentous, multinucleate, haploid ascomycete belonging to the family Pleosporaceae, order Pleosporales, class Loculoascomyces, phylum Ascomycota and kingdom Fungi. The perfect stage, *P. teres*, was first described by Drechsler (1923). The imperfect stage is *Drechslera teres* (Sacc.) Shoem. (syn.: *Helminthosporium teres* Sacc.). The pathogen was known as *H. teres* until the late 1950s when the genus *Helminthosporium* was subdivided into *Dreschlera* and *Bipolaris* based on spore morphology (Shoemaker 1959). *Pyrenophora teres* was subsequently subdivided into two forms by Smedegard-Petersen (1971) based on the distinct disease symptoms produced on barley. *Pyrenophora teres* f. *teres* produces the classic net-type symptoms while *P. teres* f. *maculata* causes spot-type lesions. The latter form was first recorded as a different species called *P. japonica* (Ito and Kuribayashi 1931). However, after successful mating between *P. teres* and *P. japonica* by both McDonald (1967) and Smedegard-Petersen (1971) it was concluded that they represented the same species.

Lifecycle and Morphology

Pyrenophora teres produces asexual spores called conidia as its primary means of reproduction. Conidia are produced singly at the end of simple conidiophores and are light, yellowish-brown in colour, generally cylindrical and are 95-120 µm long by 20 µm wide. The conidia contain 4-6 transverse septa dividing the conidium into isodiametric cells. Conidia are dispersed by air currents and are thought to travel up to seven meters within a field (Piening 1968), although Stakman et al. (1923) collected spores at an altitude of over 10,000 feet indicating dispersion can be much wider. Once

a conidium has landed on a barley host, germination will begin readily if humid conditions persist for over 24 hours and temperatures range between 20-30ºC. Germination from any of the conidial cells begins within 30 minutes to produce a hyaline coloured germ tube which differentiates within five hours to produce a terminal appressorium (Keon and Hargreaves 1983). This structure allows penetration of the epidermal layer of the barley leaf. A primary vesicle is formed within the epidermal cell after successful penetration of the outer cell wall, followed soon after by the production of a secondary vesicle. Infection hyphae then grow from the secondary vesicle to penetrate the inner cell wall of the epidermal cell. Hyphae continue to grow into the apoplastic space of the mesophyll. At no time does the hyphae penetrate any of the mesophyll cells, but at early stages of infection there is intimate contact between host and pathogen cell walls (Keon and Hargreaves 1983). These host cells show loss of tonoplast integrity, disorganization of internal membranes and eventual cell collapse (Keon and Hargreaves 1983). As infection proceeds, colonization is associated with chlorotic tissue, but close contact is no longer observed and host cell death precedes hyphae advance (Able 2003). Eventually conidiophores are produced in the necrotic tissue from which a new generation of conidia are produced. The polycyclic nature of the pathogen allows many cycles of infection to occur within one season leading to high inoculum levels. While conidia show limited viability (<3 months), mycelia within infected plants can survive for up to 15 months (Shipton et al. 1973). Infected plant residue in the field is thus considered to be the primary source of inoculum in subsequent years since fungicidal seed treatment has generally eliminated seed-borne inoculum.

Pyrenophora teres also has a sexual cycle which is thought to begin in the late summer before going dormant over winter and eventually releasing sexual spores, known as ascospores, in early spring (Shipton et al. 1973). *Pyrenophora teres* is a heterothallic, self-incompatible pathogen with two mating-types, genetically controlled by alternate alleles of the *MAT* gene (Rau et al. 2005). Formation of the sexual fruiting body, the pseudothecium, begins in autumn after the union of the male spermacia and the female ascogonium. The pseudothecium is a heavily melanized, globular structure beaked at

the apex and covered with stiff, hair-like setae (McDonald 1963). It develops under the surface of the barley epidermis before breaking through the surface at maturity. Pseudothecia range from 300-800 µm in length by 200-450 µm in width. Asci begin to develop after winter. They are bitunicate, cylindrical, contain 2-8 ascospores and are 175-275 µm long by 30-60 µm wide. There can be upwards of 50 asci within a single pseudothecium. Ascospores are light yellow, ellipsoidal, rounded at both ends and contain 3-4 transverse septa with 1-2 vertical septa in the middle cells. They are usually 36-65 µm long by 14-28 µm wide. Ascospores are not forcibly ejected, but are dispersed by wind currents. They are not considered an important source of inoculum, but are likely an important source of new pathotypes (Shipton et al. 1973).

Distribution, Host Range and Variability

Pyrenophora teres is a common pathogen throughout the temperate regions of the world where major barley producing regions are located (Dickson 1956). The net form of the disease has been observed for many years in Canada. Greaney (1944) first reported on the importance of net blotch as a seed-borne disease of barley. Severe outbreaks were described by Petersen (1956) throughout the early 1950s in the eastern prairie region of western Canada. Wallace (1960) noted that net blotch was the most severe foliar disease affecting the prairies that year and extensive losses were reported in Alberta in 1964 (Shipton et al. 1973). The spot form of the disease was first reported in Canada by Tekauz and Buchannon (1977) and was responsible for outbreaks of disease observed in Manitoba in the early 1970s. While no large outbreaks have been reported recently, net blotch continues to be a prevalent disease of concern.

Although the primary net blotch inoculum source is infested barley debris, other inoculum sources have been investigated. To date, natural foliar infections of net blotch have been observed only on *Hordeum* species (Shipton et al. 1973) and in one case in Western Australia, on *Bromus diandrus* Roth. (Khan and Boyd 1968). Kenneth (1962) showed that *P. teres* isolated from *H. murinum*, *H. murinum* ssp. *leporinum* and *H.*

marinum can infect cultivated barley. Similar results were also reported by Khan and Boyd (1968) with isolates from *B. diandrus*.

Artificial inoculation studies have revealed a wider range of potential hosts. Brown et al. (1993) tested 6 isolates of *P. teres* f. *teres* originating from *H. vulgare* and *H. murinum* ssp. *leporinum* on 95 species covering 16 genera in the Poacea family. Sixty-five species were observed as hosts, 27 were previously reported *Hordeum* species or *B. diandrus* (Shipton et al. 1973), but 38 were new host species from the genera *Cynodon*, *Deschampsia*, *Hordelymus* and *Stipa*.

Part of the difficulty dealing with net blotch is extensive pathotype variability. Pon (1949) was the first to report variability in *P. teres* pathogenicity. McDonald and Buchannon (1962) first described the presence of net blotch pathotypes in Canada, which was again reported by Tekauz and Mills (1974). Tekauz (1990) undertook the first extensive survey of pathotypes in western Canada. Forty-five net form and 20 spot form pathotypes were recorded using nine and 12 differentials, respectively. A similarly large number of pathotypes were recorded in the USSR by Afanasenko and Levitin (1979) who identified 80 net form pathotypes with seven differential lines. Steffenson and Webster (1992) carried out an assessment of California net form diversity and found 13 pathotypes using 22 differential lines. *Pyrenophora teres* pathotype variability does not appear to be as large in other regions of the world. In Western Australia, Gupta and Loughman (2001) noted only two net form and two spot form pathotypes across 47 differentials, while Platz et al. (2000) found 13 net form pathotypes in the rest of Australia using 15 differentials. Similarly, only four pathotypes were identified using four differentials in Egypt (El-Fahl et al. 1982).

The relative frequency of net or spot form isolates in fields is not well defined. Most studies report only on the occurrence of net form isolates. Part of the reason for this may be the difficulty in visually distinguishing spot form symptoms from lesions typical of spot blotch, caused by *Cochliobolus sativus*. Thus, reports on spot form may under represent true levels due to misdiagnosis when collecting samples from the field. With

this in mind, Tekauz (1990) reported that of the 219 isolates collected, 18% were spot form and 72% were net form. Gupta and Loughman (2001) found only five spot form isolates out of 79 isolates collected in Western Australia, while Steffenson and Webster (1992) reported no spot form isolates in California to that point in time.

Pyrenophora teres variability has also been documented with molecular data. Peever and Milgroom (1994) examined the diversity among and within five populations of *P. teres* originating from Canada, the USA and Germany. They found total genetic variability split nearly equally between inter-(46%) and intra-(54%) population variability. The high level of interpopulation variability likely arose when a limited number of isolates were introduced to a region on infected seed, followed by restricted migration between growing areas. It was also noted that all populations shared common bands indicating all originated from a single founder population, likely from the Middle East where the pathogen co-evolved with barley. A high percentage of RAPD loci were randomly associated within four of the five populations indicating random sexual reproduction was occurring.

Campbell et al. (2002) found a high level (63%) of diversity between net and spot form populations. More significantly, a dendrogram produced from the RAPD data showed that the isolates clustered predominantly based on form, with spot and net isolates producing two distinct clusters. Six isolates produced from a spot by net form cross produced a separate clade in the dendrogram that associated with three isolates that did not group with the two major clades. This suggests that limited natural intermating between types can occur.

Rau et al. (2003) provided stronger evidence that sexual reproduction is rare or absent between spot and net forms in the field. They collected 150 isolates (45% net, 55% spot) from five areas of Sardinia and screened them with AFLP markers. They also found the net and spot forms separated into two distinct clades with no intermediate clades and very few common bands between the two forms.

While the molecular data presents a picture of substantial diversity, it can not be translated directly into an equal number of pathotypes. However, evidence that sexual reproduction is common, and along with mutations and gene rearrangements associated with such events, means that new combinations of genes are continually being produced resulting in potential new pathotypes.

Toxins

The production of toxins by *P. teres* was thought to account for the chlorosis and water-soaking symptoms observed on susceptible barley lines. Histological studies of infected leaves showed symptoms developed in advance of penetrating hyphae, indicating the presence of a diffusable substance such as a toxin. Additionally, culture filtrates applied to excised barley leaves produced symptoms similar to those produced by the pathogen (Smedegard-Petersen 1977a).

This was proven when two toxins were isolated from *P. teres* cultures and infected leaves (Smedegard-Petersen 1977a). Named toxin A and toxin B, they produced some key symptoms of net blotch, such as chlorosis, necrosis and water-soaking, but they did not produce the net or spot symptoms. It was also observed that the most virulent isolates tended to produce the highest levels of toxins. The range of host species on which symptoms could be elicited by either the pathogen or the toxin was similar, but the toxins were able to cause symptoms on additional hosts.

Subsequent work by Bach et al. (1979) characterized toxin A as L,L-N-(2-amino-2-carboxyethyl) aspartic acid and toxin B as anhydroaspergillomarasmine A (1-(2-amino-2-carboxyethyl)-6-carboxy-3-carboxymethyl-2-piperazinone). They also identified a third toxin, toxin C, as aspergillomarasmine A (N-[2-(2-amino-2-carboxyethyl-amino)-2-carboxyethyl] aspartic acid). This toxin has been isolated from other fungal pathogens such as *Aspergillus flavus* f. sp. *oryzae* (Haenni et al. 1965), *Colletotrichum gloeosporoides* (Bousquet et al. 1971) and *Fusarium oxysporum* f. sp. *melonis* (Camporota et al. 1973). Friis et al. (1991) used radioisotopes to determine that toxin A

is a precursor of toxin C, whereas toxin B is likely an artefact because it is formed from toxin C by a non-enzymatic conversion at low pH. Thus, *P. teres* appears to produce only one toxin.

Toxin A and C caused chlorosis and necrosis on barley leaves at levels much lower than toxin B (Friis et al. 1991; Smedegard-Petersen 1977a). Toxin C is able to disrupt the water balance in plant cells and its activity is enhanced by the presence of iron III which may allow the formation of toxic metal chelates (Gaumann 1951). The weak toxicity of toxin B is due to its ring structure which does not allow it to chelate iron. The highly toxic nature of toxin C and the high levels to which it accumulates indicates it likely plays a major role in producing the disease symptoms incited by *P. teres*.

Interest in these toxins stems from their potential application as a simple method of evaluating barley germplasm for resistance. Initial work showed some correlation between sensitivity of detached barley leaves to the pathogen alone or to partially purified toxins (Sharma 1984; Smedegard-Petersen 1977a). However, the presence of other metabolites in the filtrates could also cause damage unrelated to virulence. Therefore, Weiergang et al. (2002b) evaluated *P. teres* isolates and culture conditions which would allow purification of high concentrations of pure toxins. Using these methods, detached leaves from 25 barley lines were subsequently evaluated for their reaction to purified toxins (Weiergang et al. 2002a). This data was correlated to infection by both spot and net form strains of *P. teres*. They found that toxin A caused primarily chlorotic symptoms with little necrosis, while toxin C-treated leaves showed mainly necrosis with little chlorosis. Once again toxin C was effective at the lowest concentrations, followed by toxin A. Toxin B was unable to elicit any symptoms at the concentrations used for toxins A and C. They found that using 0.25 mM toxin C or 0.75 mM toxin A and evaluating the symptoms 120 hours after treatment showed the best differentiation between the barley lines and showed significant correlation to inoculation with the pathogen alone. The authors concluded that toxin screening of barley lines at early generations of a breeding program is a feasible way of screening for resistant material. Unfortunately correlations were calculated with isolate data grouped together,

not allowing an analysis of individual isolate correlations. In addition, depending on which toxin and which barley type (6-row or 2-row) was being analysed, correlations ranged from 59% to 85%, meaning there would be a number of incorrect assessments in a screening program.

Reiss and Bryngelsson (1996) also noted that, while toxin application to detached leaves mimicked net blotch symptoms, cultivar-specific resistance was lost, indicating that resistance is not due to resistance to the toxin. This is a significant shortcoming of using toxins for evaluation of barley resistance to *P. teres* and supports the idea that the *P. teres*-barley pathosystem likely does not conform to the "toxin" model.

The Host: Barley Resistance to *Pyrenophora teres*

Historical Screening of Germplasm

Studies attempting to identify sources of barley resistant to net blotch began early in the 20th century. However, the first comprehensive search for resistant germplasm did not occur until Schaller and Wiebe (1952) screened >4,500 accessions from the world barley collection in the late 1940s. After testing these accessions with a mixture of Californian isolates, they identified 75 resistant lines, 61 of which originated from Manchuria. Subsequent screening of an enlarged world barley collection (>6000 accessions) by Buchannon and McDonald (1965) using single and mixed inoculum from western Canada identified 40 resistant lines, with 20 originating from North Africa and Ethiopia.

The ability of *P. teres* to infect wild species of *Hordeum* has also led to investigations of resistance in these species. This approach to identifying novel sources of resistance has proven fruitful with other barley diseases. For example, the *Mla-6* and *Mla-14* powdery mildew resistance genes were introduced into cultivated barley from *Hordeum vulgare* ssp. *spontaneum* (Jorgensen 1992). Sato and Takeda (1997) screened over 300 wild

Hordeum accessions for resistance to four net form isolates. Over half the accessions were *H. vulgare* ssp. *spontaneum* with significant numbers of *H. bulbosum*, *H. murinum* and *H. marinum*. Many resistant lines were identified in all species, but reproductive barriers makes transferring the resistance genes to cultivated barley difficult. However, *H. vulgare* ssp. *spontaneum* crosses readily with cultivated barley and within this species, many accessions from Afghanistan showed high levels of resistance to most isolates.

Genetic Studies of Resistance

A map of the barley genome indicating all the loci associated with net blotch resistance is provided in Figure 1.1. Schaller (1955) first explored the genetic basis of resistance to net blotch. Using a mixture of isolates from California, a single major resistance (*R*) gene (*Pt1*) was identified in the Manchurian line *Tifang* (CI 4407-1). Mode and Schaller (1958) also used mixed inoculum and evaluated several populations with resistance derived from Manchurian lines to identify two more major resistance genes, *Pt2* and *Pt3*. *Pt2* was identified in *Ming* (CI 4797), *Harbin* (CI 4929) and *Manchuria* (CI 2335) and was linked (2.6% recombination) to *Pt1*. *Pt3* was found in CI 4922 and CI 2750 and was not linked to the other genes. Khan and Boyd (1969) were unable to find recombination between *Pt1* and *Pt2*, even within a large population, but identified a new gene (*Pta*) responsible for resistance to a Western Australian isolate in Tifang, Ming, Manchuria, CI 5791 and CI 9819.

Bockelman et al. (1977) used *Betzes* primary trisomics in crosses with several resistant lines to identify four resistance genes and assign them to specific barley chromosomes. *Rpt1a* was identified on chromosome 3 in Tifang, *Rpt3d* was located on chromosome 2 in CI 7584 and *Rpt1b* and *Rpt2c* were found in CI 9819 on chromosomes 3 and 5, respectively. Whether these genes represent the same ones identified previously is difficult to determine since different inoculum sources were used and no chromosome assignments were made in the earlier work.

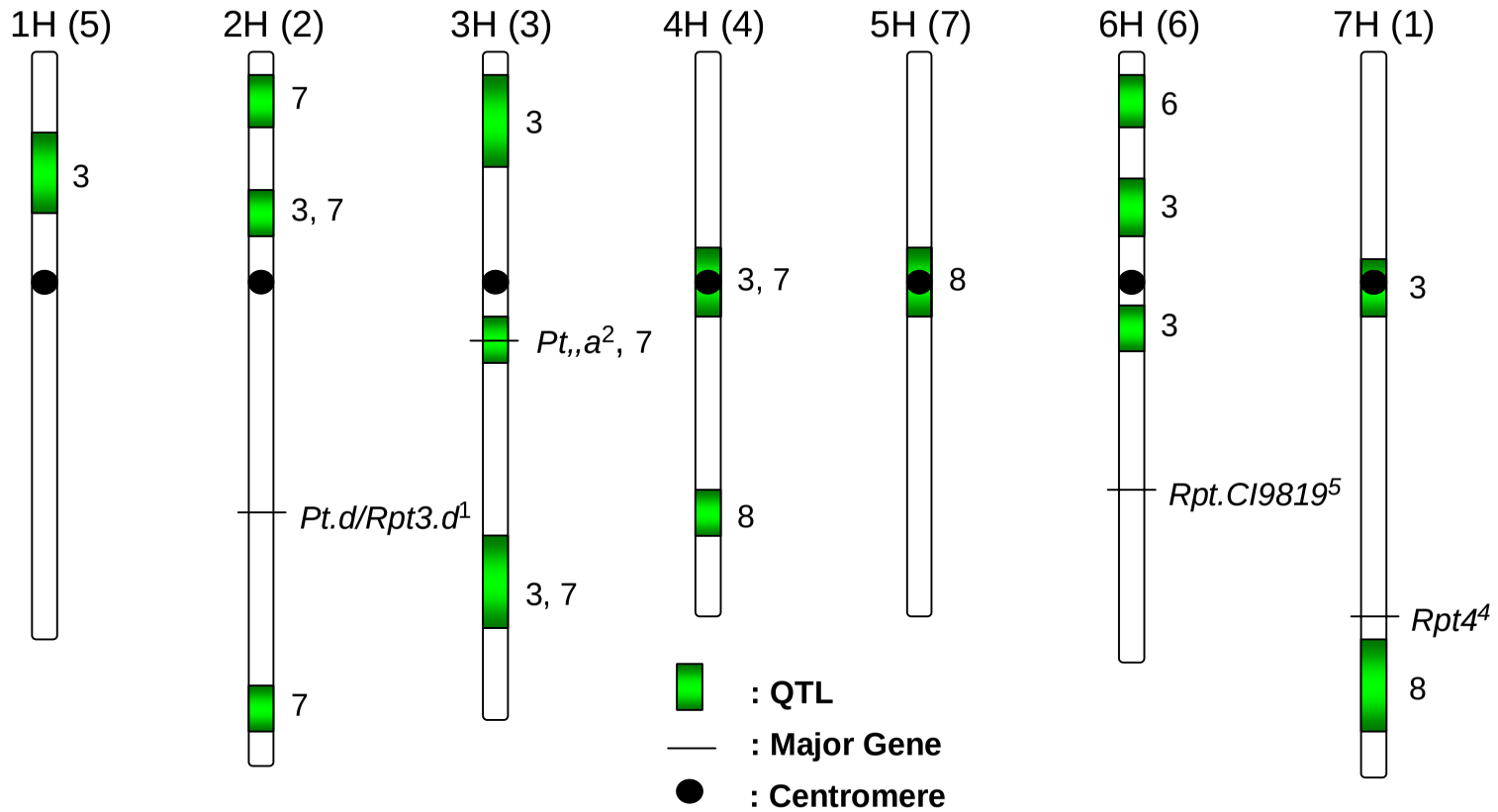

Figure 1.1. Location of resistance genes and regions within the barley genome associated with net blotch resistance. Approximate positions are indicated since mapping was done in different populations with no reference to a common map. Numbers to the right of QTL regions and subscripted in gene names indicate the study in which resistance was identified. 1. Bockelman et al. (1977), 2. Graner et al. (1996), 3. Steffenson et al. (1996), 4. Williams et al. (1999), 5. Manninen et al. (2000), 6. Cakir et al. (2003), 7. Raman et al. (2003), 8. Williams et al. (2003).

All initial studies on net blotch resistance concentrated on either seedling resistance evaluated in the greenhouse, or on adult resistance as evaluated in the field. Tekauz (1986) was the first to explore plant age as a factor in net blotch resistance. Using five single-spore isolates and 12 barley lines he found resistance was either maintained or improved as the plant matured. This was true for lines that were either initially susceptible or resistant. Similar results were reported in subsequent studies (Gupta et al. 2003; Tekauz 2000). Douiyssi et al. (1998) observed somewhat different results with two Moroccan isolates inoculated on 38 barley lines. Resistance to one isolate tended to increase with plant age, but the opposite was observed with the second isolate. This is the only report of such an interaction, but if it proves to be more common it presents a challenge to breeders attempting to provide resistance throughout all plant development stages.

A more comprehensive attempt to understand the genetic basis of this phenomenon was made by Steffenson et al. (1996). Using a single net form isolate they conducted a quantitative trait loci (QTL) analysis on a doubled haploid (DH) population inoculated at the seedling stage (14 days) and at mid-tillering. They identified three QTL for seedling resistance on chromosomes 4 and 6 and seven QTL for adult resistance on all chromosomes except chromosome 5. There was only one region that conferred resistance at both stages, however its influence decreased with plant age. It appears from this study that while overall resistance is maintained, different genes are activated or repressed depending on plant age.

Richter et al. (1998) conducted another QTL study on seedling resistance using one isolate of net blotch. They individually scored the first and second leaves at day seven and day nine after inoculation. Twelve QTL were mapped (three from each leaf-time combination) in total. The QTL associated with the second leaf mapped to similar regions on chromosomes 3, 4 and 6 as those reported by Steffenson et al. (1996) for adult resistance. Additional QTL on chromosomes 1, 2, 4 and 6 were identified for first leaf resistance. No overlapping QTL were detected, again suggesting an age-related response to net blotch.

A number of groups have also mapped major resistance genes to well defined loci. Graner et al. (1996) were first to map a resistance gene, *Pt,,a*, to the proximal portion of the long arm of chromosome 3. This gene conferred resistance against a Canadian net form isolate. *Rpt4* was mapped to the long arm of chromosome 7 and provided resistance to a mixed inoculum of spot form isolates (Williams et al. 1999). Manninen et al. (2000) used a mixture of four Finnish net form isolates to identify a major gene on chromosome 6, in the same region as a QTL for seedling resistance (Steffenson et al. 1996).

Several recent QTL studies by Australian groups have extended and confirmed previous regions showing resistance to net blotch. Williams et al. (2003) confirmed the importance of the *Rpt4* locus for seedling resistance to spot form net blotch in four different lines and demonstrated that adult plant resistance was located on chromosomes 4 and 5, as well as, to a region distal to *Rpt4* on chromosome 7. Cakir et al. (2003) analyzed resistance in two populations, one screened with five net form isolates and the other with one. One major gene located on chromosome 6 was associated with seedling resistance to all isolates in both populations. Two other QTL were detected on chromosomes 2 and 3 in one population. The same major gene was also important for adult resistance. This gene appeared to be located in the same region as the major gene reported by Manninen et al. (2000) and a QTL identified by Steffenson et al. (1996), however the lack of common markers between these studies does not allow a firm conclusion. A study by Raman et al. (2003) analyzed seedling resistance in four populations using two net form isolates. Five QTL were mapped to chromosomes 2, 3 and 4 and corresponded to regions already determined important for net blotch resistance (Graner at al. 1996; Steffenson et al. 1996).

Other Aspects of Resistance

A number of studies have investigated the processes involved in mounting a resistance response to net blotch. Keeling and Bantarri (1975) examined macroscopic and

histological differences between susceptible and resistant interactions. They observed no differences in spore germination, germ tube growth or successful penetration attempts. Germ tube growth was inhibited more frequently in resistant lines than in susceptible barley lines only after penetration. Keon and Hargreaves (1983) noted that even for compatible reactions there were many unsuccessful penetration attempts. These repulsed attempts were associated with the formation of papillae below the penetration peg. Papillae were also observed by Lyngs Jorgensen et al. (1998) in response to penetration attempts and were observed more frequently on barley leaves induced to be more resistant by preinoculation with non-host pathogens.

Reiss and Bryngelsson (1996) examined gene expression during pathogen attack in barley leaves and found a large number of common genes induced by *Puccinia hordei*, *E. graminis* and *P. teres*. They identified a number of pathogenesis-related (PR) proteins such as peroxidases, β-1,3-glucanases, chitinases, PR-1a and 1b and several thaumatin-like proteins (PR-5 family). A subsequent study by Reiss and Horstmann (2001) identified eight thaumatin-like proteins expressed in barley in response to *P. teres* infection.

A novel nuclear-targeted protein, HVs40, which may play a role in signalling during the hypersensitive response (HR) was identified in barley leaves (Krupinska et al. 2002). It was expressed during senescence and in leaves undergoing chlorosis in response to *P. teres* infection. An increasing number of studies are finding considerable overlap in genes expressed during HR and senescence (Quirino et al. 2000) and that both processes respond to salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). *HVs40* was also stimulated by SA and JA which led the authors to propose that reactive oxygen species (ROS) produced during infection stimulate JA production which turns on the *HvS40* gene eventually leading to the HR.

ROS, such as superoxide and hydrogen peroxide, are produced during the initial oxidative burst, along with the HR, as part of the gene-for-gene resistance reaction. ROS are also thought to promote the HR in adjacent cells. Able (2003) explored the role

of ROS during *P. teres* infection by comparing the reaction of the barley cultivar 'Sloop' to virulent and avirulent isolates. ROS could only be detected in the susceptible interaction. Superoxide was produced in cells adjacent to the hyphae early in the infection and as infection progressed was found in cells further away from the hyphae. At least some of the superoxide was produced by the pathogen at levels known to induce cell death (Able et al. 1998). However, there was no correlation between concentrations produced by the pathogen and virulence. Hydrogen peroxide was detected later in the response and in the mesophyll further from the hyphae. The production of ROS in advance of hyphal growth suggests programmed cell death (PCD) is occurring and confirms the previous observation that chlorosis occurs in advance of hyphae. Toxin C produced by *P. teres* may be responsible for the induction of PCD, and the associated presence of ROS, since induction of PCD is a common mode of action associated with toxins (as will be described later). Alternatively, ROS themselves may act as a diffusable signal to initiate PCD. Interestingly, six times more superoxide dismutase (SOD) activity was detected in resistant reactions, indicating that this antioxidant may suppress further induction of HR by removing the pool of ROS.

Pathogen Parasitism and Plant Responses

Plant pathogens can be broadly divided into three groups based on the form of host parasitism they adopt to complete their lifecycle. At one extreme are obligate biotrophs which require living host cells to grow and reproduce. They form intimate associations with the host cells through the formation of haustoria. Extensive exchange of metabolites across the plasma membrane of haustoria and host cells occurs without causing plant cell death. Pathogens such as *Peronospora parasitica* (an oomycete), the cause of downy mildew on *Arabidopsis*, and *Melampsora lini* (a fungus), the cause of flax rust, are such specialized pathogens in that they cannot be cultured on artificial media. Other biotrophs such as *Cladosporium fulvum*, the cause of tomato leaf mold,

still require live host cells to acquire nutrients, but do not form haustoria and can be cultured outside the plant.

At the other extreme are necrotrophic pathogens which kill host cells before colonizing the tissue and feeding on released nutrients. The production of toxins is often associated with and essential for these pathogens. Fungi such as *Botrytis cinerea*, the cause of grey leaf mold, and host-selective toxin (HST)-producing species like *Alternaria alternata* are examples of necrotrophs.

Between these two extremes are the hemibiotrophs which tend to act as biotrophs in the early stages of infection, but later kill host cells like necrotrophs. Fungi such as *P. teres* and *Magnaporthe grisea*, the rice blast pathogen, grow for a time in the intercellular spaces of the plant, but eventually the action of toxins and avirulence (Avr) proteins kill the infected plant cells. Bacterial pathogens such as *Pseudomonas syringae* can also be considered hemibiotrophic since they also grow and reproduce in the intercellular space before injecting a range of Avr proteins and toxins into plant cells.

The contrasting requirements of these pathogenic lifestyles have resulted in different defense strategies being employed by plant hosts. Gene-for-gene resistance, and the resulting form of PCD known as the HR, has not been described for necrotrophic pathogens and evidence suggests that this type of resistance is not effective. In fact, several studies have shown that induction of PCD by necrotrophs, such as *B. cinera*, is part of its virulence activity and actually promotes necrotrophic growth (Govrin and Levine 2000). High levels of ROS such as hydrogen peroxide, which form part of the HR and are toxic to biotrophs and hemibiotrophs, enhance *B. cinerea* growth. Similarly, induction of the HR by pre-inoculation with avirulent strains of *P. syringae* increases susceptibility to *B. cinera* infection (Govrin and Levine 2000). Not surprisingly, mutation of genes required for SA signalling (a downstream result of the HR) do not affect resistance to *B. cinera* (Ferrari et al. 2003), but exogenous application of SA prior to infection does reduce lesion size suggesting there may be some role for SA signalling responses (Zimmerli et al. 2001). However, mutations in the JA (Thomma et al. 1998)

and ET signalling pathways (Ferrari et al. 2003) clearly reduces resistance to these pathogens. It is interesting to note that the JA/ET pathways are considered quite separate from the SA pathway in that they induce a different set of genes and show mutual negative regulation (Glazebrook 2005). This aspect of resistance will not be discussed further, but the HST section will touch briefly on how these compounds benefit necrotrophic pathogens.

In contrast, gene-for-gene resistance and the HR is an effective strategy to limit the growth of biotrophic and hemibiotrophic pathogens (Glazebrook 2005). This response is thought to restrict the availability of water and nutrients to the pathogen. The activation of SA signalling and systemic acquired resistance (SAR) by the HR are also critical aspects of resistance against these pathogens. Details of the gene-for-gene interaction and related topics will be discussed in some detail because this form of resistance plays a key role in the *P. teres*-barley pathosystem.

Basal Defense

Of the thousands of species of bacteria, fungi, oomycetes and viruses which are plant pathogens, only a relatively small proportion are capable of infecting any one particular plant species. The collection of passive and induced defenses that are responsible for repelling a significant number of potential pathogens is known as non-host resistance (Kim et al. 2005). Passive defenses, such as the cell wall and pre-formed antimicrobial compounds (phytoanticipins), represent the first barrier potential pathogens encounter. The induced portion of non-host resistance is known as the basal defense system and it forms one part of a plant's innate immune system, the other being the *R* gene-mediated defense system (Jones and Takemoto 2004). This growing field of research has recently established key components of the basal defense system and has revealed significant similarities to the better known *R* gene-mediated defense system. Additionally, evidence

is accumulating that pathogen avirulence proteins target this system as part of their strategy to gain access to the plant host (Kim et al. 2005).

Induction of the basal defense system depends on recognition of a range of compounds produced by pathogens, collectively termed pathogen associated molecular patterns (PAMPs). PAMPs are molecules that are indispensable for pathogenicity, unique to pathogens and conserved across many pathogen species (Navarro et al. 2004). These characteristics make PAMPs ideal cues to a plant that a foreign body is present. Examples include chitin, glucans and glycoproteins from fungi and lipopolysaccharides and flagellin from bacteria (Gomez-Gomez and Boller 2002; Montesano et al. 2003). In addition to structural components, enzymes such as xylanase and endopolygalacturonase can also induce plant defenses directly or via plant cell wall-derived enzymatic products (Boudart et al. 2003; Poinssot et al. 2003).

Insight into PAMP recognition and the subsequent events leading to a defense response is more recent. Gomez-Gomez and Boller (2000) identified FLS2 (flagellin insensitive 2), a protein responsible for recognizing the bacterial flagellin protein. FLS2 belongs to a class of proteins known as receptor-like kinases (RLKs), membrane-bound proteins that contain an extracellular leucine-rich repeat (LRR) and a cytoplasmic protein kinase (Figure 1.2). This RLK is strikingly similar to Toll-like receptors which are key to PAMP perception in mammals and to a group of plant R proteins exemplified by Xa-21 from rice and the Cf family in tomato. A second PAMP receptor, LeEix (*Lycopersicon esculentum* ethylene-inducing xylanase), identified from tomato, recognized a 22 kD ethylene-inducing fungal xylanase (Ron and Avni 2004). LeEix was structurally similar to FLS2, except that no kinase domain existed, and it was thus classified as a receptor-like protein (RLP) (Figure 1.2).

Perception of PAMPs induces a number of plant defense mechanisms that have become characteristic of the basal defense system. These include cell wall changes, activation of signalling pathways and production of antimicrobial compounds. Recognition of flg22, the 22 amino acid peptide of flagellin recognized by FLS2, leads to callose deposition

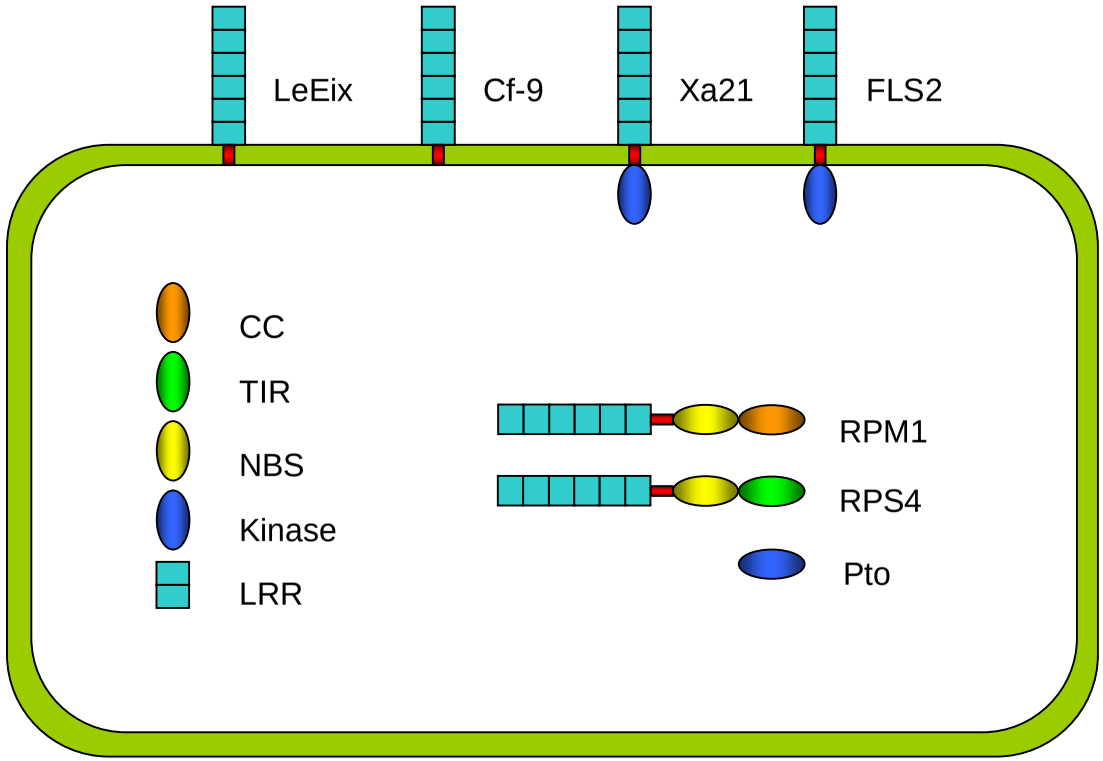

Figure 1.2. Protein structural domains common in many basal defense receptors and resistance gene proteins. LeEix and FLS2 are PAMP receptors which recognize a fungal xylanase and a bacterial flagellin peptide, respectively. Pto is a class 1 resistance protein from tomato that recognizes the AvrPto effector from *Pseudomonas syringae*. RPM1 is a class 2 resistance protein from *Arabidopsis* that recognizes AvrRpm1 and AvrB from *Pseudomonas syringae*. *L* are a group of class 3 resistance proteins from flax that recognize the AvrL567 effectors from *Melampsora lini*. Cf-9 is a class 4 resistance protein that recognizes the Avr9 effector from *Cladosporium fulvum* and, Xa21 is a class 5 resistance protein from rice that has an undefined effector protein. Adapted from Jones and Takemoto (2004).

(Gomez-Gomez et al. 1999), PR protein production and activation of mitogen-activated protein kinase (MAPK) signalling (Asai et al. 2002) (Figure 1.3). Similar responses are observed with two *Phytophthora* PAMPs. Pep-13, a 13 amino acid fragment from a 42 kDa cell wall transglutamase, and NPP1 (necrosis-inducing *Phytophthora* protein 1), a 24 kDa cell wall protein, induce MAPK signalling leading to callose deposition, PR production, ROS generation and in the case of NPP1, HR-like cell death (Fellbrich et al. 2002) (Figure 1.3).

An interesting feature of these downstream responses is their commonality with *R* gene-mediated defenses. This is not surprising given the structural similarity between the receptors mediating these two parts of the immune response (Figure 1.2). Both tobacco *N* and tomato *Cf-9 R* genes induce MAPK pathways upon recognition of the TMV and *Cladosporium fulvum* AVR9 protein, respectively (Romeis et al. 1999; Zhang and Klessig 1998). Other downstream proteins required for *R* gene defense, such as PAD4 (phytoalexin deficient 4) and NDR1 (non-race-specific disease resistance 1), are also necessary for NPP1-mediated PR expression (Fellbrich et al. 2002). Navarro et al. (2004) demonstrated that 13 of 17 genes upregulated during AVR9-Cf9 interaction were also upregulated by flg22 perception by FLS2.

Despite the efficiency of the basal defense system, pathogenic species have developed a variety of specialized compounds able to circumvent it. These are known as either avirulence or effector proteins and display a diversity of methods by which they not only disrupt the basal defense system, but also resistance gene-mediated defense (Abramovitch and Martin 2004). In response to avirulence proteins, plants have developed another line of defense-mediated by resistance genes which are able to recognize these proteins. This part of the innate immune system differs from the basal defense system primarily in the timing and intensity of the response, with *R* gene reactions being more rapid and intense (Kim et al. 2005). These subjects will be discussed in the following sections.

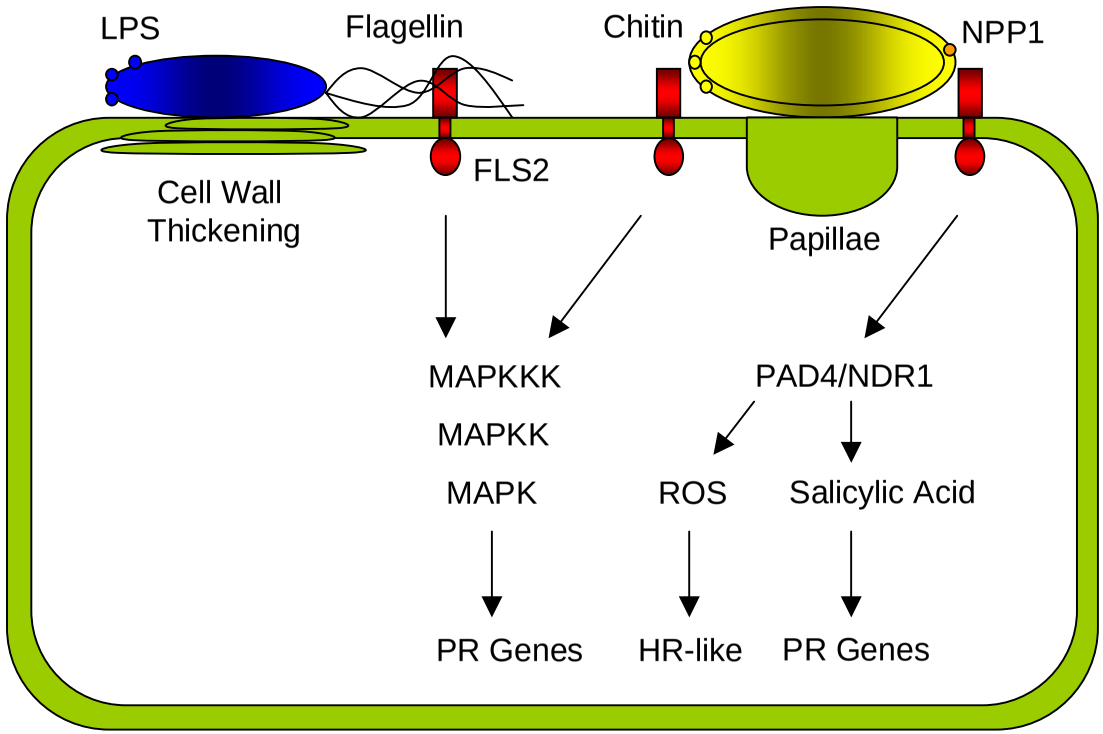

Figure 1.3. Common basal defense pathways and responses induced by PAMPs. Common PAMPs such as LPS and flagellin from bacteria and fungal chitin and NPP1 molecules are indicated. PAMP receptors such as FLS2 are indicated. Adapted from Nomura et al. (2005).

Avirulence Genes

Gene-for-Gene Hypothesis

Earliest studies attempting to understand the interactions between plants and fungal pathogens can be traced back to H.H. Flor. In his classic papers (Flor 1946; Flor 1947) using the flax rust pathogen (*Melampsora lini*), it was demonstrated that host resistance was often conditioned by single, dominant genes while single, recessive genes accounted for virulence in the pathogen (Figure 1.4). This ultimately led to the gene-for-gene hypothesis which stated host-pathogen interactions were conditioned by pairs of genes, one from the host (resistance genes) and one from the pathogen (avirulence genes) (Flor 1955). This basic concept has served as the fundamental principle for this research area for the past fifty years.

The idea of having dominant genes in a pathogen which limit virulence presented a conceptual dilemma. However, many studies involving fungi, bacteria, viruses and insects have confirmed this concept (Agrios 1997). This led to the concept that the "active" *Avr* gene was superimposed upon a basic compatibility between the host and pathogen, while the alternate allele allowed the virulent state (Ellingboe 1996). Opponents of this theory stated that according to Darwinian selection theory, it was unlikely that nature would select for genes detrimental to species' survival (Person and Mayo 1974). The predominant view until the 1980s was that avirulence was similar to microbial auxotrophy in that the pathogen lacked something required to infect the host in the same way bacteria lack an allele to grow on a minimal media (Day 1974).

It was not until the first *Avr* gene was cloned in the early 1980s from the bacterial pathogen *Pseudomonas syringae* pv. *glycinea* (Staskawicz et al. 1984), the causal agent of soybean bacterial blight, that the concept of pathogens carrying genes which limit their virulence began to be accepted. Since then over 50 *Avr* genes from bacterial,

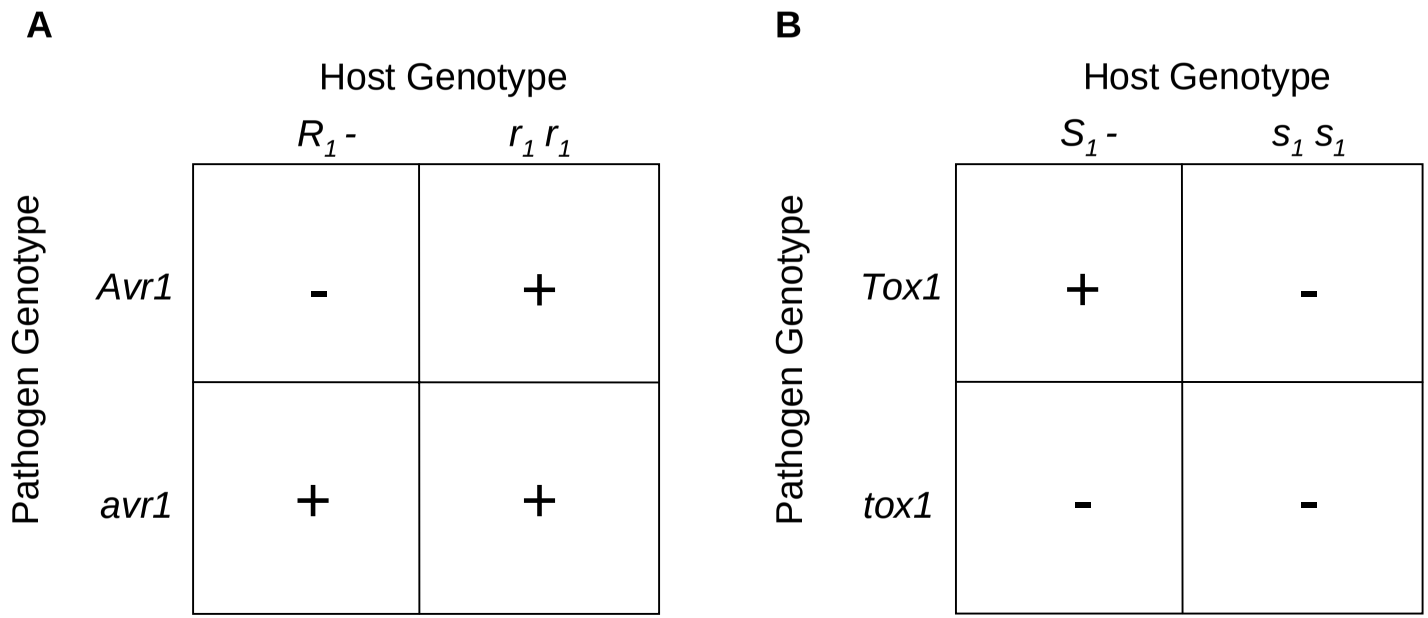

Figure 1.4. Models of plant-pathogen interactions. (A) The gene-for gene model where incompatibility forms the basis of specificity and, (B) the toxin model where compatibility forms the basis of specificity. *Avr/avr*: avirulence genes, R_1/r_1: resistance genes, *Tox/tox*: toxin producing genes, S_1/s_1: susceptibility or toxin receptor genes. +: indicates a compatible (or susceptible) reaction, -: indicated incompatible (or resistant) reaction. Adapted from Gabriel (1999a).

fungal and oomycete pathogens have been cloned, providing insight into this class of genes.

Avirulence Gene Function I - Fitness

The first function ascribed to *Avr* genes, other than pathogen perception, was a role in pathogen fitness. Fitness can be generally described as the ability of a pathogen to infect, grow and disseminate successfully, and is quantifiable using criteria such as multiplication rate, infection efficiency and symptom expression. A fitness function was first demonstrated by Kearney and Staskawicz (1990) with the *avrBs2* gene from *Xanthomonas campestris* pv. *vessicatoria*, the causal agent of bacterial spot in pepper and tomato. Pepper plants containing the *Bs2* resistance gene produce the HR when infected with *X. campestris* strains containing the *avrBs2* gene. However, induced and natural mutations of the *avrBs2* gene not only resulted in loss of the HR response in *Bs2* containing plants, but a reduced rate of bacterial growth was observed on plants lacking *Bs2*, implying a fitness function associated with the *avrBs2* gene. Chang et al. (2000) also observed that *P. syringae* pv. *tomato* strains carrying the *avrPto* gene showed enhanced growth and necrosis on susceptible tomato lines lacking the *Pto* (*Pseudomonas syringae* pv. *tomato*) resistance gene. Similarly, the presence of the *avrRpt2* gene in *P. syringae* pv. *tomato* strains promoted 50-100 fold more growth on *Arabidopsis* lines lacking the corresponding *RPS2* (resistance to *Pseudomonas syringae* pv. *tomato* 2) resistance gene (Chen et al. 2000).

An important paper by Bai et al. (2000) demonstrated that *Avr* genes were not all created equal. They noted that three *Avr* genes from *X. oryzae* pv. *oryzae* affected aggressiveness to varying degrees. By mutating *avrXa7*, *avrXa10* and *avrxa5* individually, or in combination, and monitoring pathogen growth on susceptible rice lines, they observed that loss of *avrXa7* caused the greatest decrease in aggressiveness, as demonstrated by decreased lesion size and bacterial growth, while at the other extreme *avrXa10* had no effect on fitness.

Based on such observations Vera Cruz et al. (2000) proposed it would be possible to predict the durability of *R* genes using the contribution an *Avr* gene makes to aggressiveness as an indicator. They reasoned that if an *Avr* gene contributed significantly to pathogen growth then loss of that gene would impose a significant fitness penalty and would be unlikely to occur. Therefore, any resistance gene targeted against such an *Avr* gene would likely be durable because the pathogen would be unable to function without the *Avr* gene. To test this theory they conducted a three year field trial using near isogenic rice lines each containing a single resistance gene (*Xa7*, *Xa10*, *Xa4*). Based on fitness loss observed in the lab when *X. oryzae* pv. *oryzae* strains carrying each corresponding *Avr* gene were mutated, they predicted that the *Xa7* gene would be the most durable and the *Xa10* gene the least. They observed that *Xa10* lines showed 100% disease incidence and >40% diseased leaf area, while *Xa7* lines showed <5% disease incidence and <1% diseased leaf area. Strains which were able to grow on *Xa7* lines had either completely or partially lost *Avr* function. As *Avr* function decreased so did aggressiveness, in fact, strains that had completely lost the gene did not persist in the population. Interestingly, strains with partial loss of the gene were still aggressive, but never produced a severe disease outbreak. This significant finding not only provided an explanation for the durability of some single disease resistance genes such as *Rpg1* for stem rust in barley and *Lr34* for leaf rust in wheat, but also demonstrated durability could be evaluated before widespread incorporation into cultivars.

It was evident that some *Avr* genes played a critical role in the lifecycle of a pathogen, thus null mutations of such genes were not a viable option for the pathogen to avoid the host defense system. However, the study by Vera Cruz et al. (2000) indicated that pathogen recognition and fitness function within the *Avr* gene could be separated. Recently, Yang et al. (2005) demonstrated that a series of natural and induced deletions in the central repeat region or near the carboxy (C)-terminal of the AvrXa7 protein were responsible for this pathogen phenotype. Similarly, a series of nonsense mutations in the *avrRpt2* gene, resulting in C-terminal deletions of AvrRpt2, prevent induction of *RPS2*-mediated HR in *Arabidopsis*, but do not affect virulence of the pathogen (Lim and Kunkel 2004). As mentioned above, the *avrPto* gene enhances bacterial growth on

tomato lines lacking *Pto*. Shan et al. (2000) identified three amino acid substitutions in AvrPto that abolish binding to Pto. These changes destroy the AvrPto avirulence function yet maintain its virulence capability. Six other point mutations located outside this region abolished both virulence and avirulence function. The ability to lose *R* gene recognition, either with or without maintenance of the virulence function, has been observed with other *Avr* genes and will be noted in subsequent sections. These studies reveal how pathogens can adapt to the deployment of new resistance genes and may provide valuable insights to explain *R* gene durability.

Avirulence Gene Structure and Cellular Localization

The cloning and sequencing of more and more bacterial *Avr* genes revealed many contained characteristic protein motifs critical for function and indicated their activity occurred within the plant cell. The first group of such *Avr* genes contained nuclear localization signals (NLS) and acidic transcriptional activation domains (AD) and is exemplified by the *avrBs3* gene family found in many xanthomonad species. AvrBs3 proteins have 90-97% amino acid identity (Gabriel 1999b) and all contain typical eukaryotic NLS (Yang and Gabriel 1995) and AD (Zhu et al. 1998) located in the C-terminal. Not surprisingly, the AvrBs3 protein from *X. campestris* pv. *vesicatoria* was detected by antibody labelling inside the plant cell (Szurek et al. 2002) providing the first direct evidence that avirulence gene products do enter plant cells. On susceptible plants these genes induce hypertrophy in mesophyll cells. Disruption of the NLS inhibits the induction of hypertrophy symptoms (Marois et al. 2002) and localization to the nucleus (Szurek et al. 2002). It is hypothesized that AvrBs3 is able to affect these changes because it is a transcription factor, a concept supported by the fact that the AvrBs3 homologue, AvrXa7, directly binds to AT rich DNA sequences (Yang et al. 2000) and upregulates auxin-like and expansin genes involved in cell enlargement (Marois et al. 2002).

The second group of internally targeted *Avr* genes are found in a number of pseudomonads. This group contains myristoylation motifs which typically target

proteins to the plasma membrane. Nimchuk et al. (2000) demonstrated that AvrRpm1 and AvrB from *P. syringae* pv. *maculicola* contain amino (N)-terminal myristoylation sites which indeed targeted these proteins to the plasma membrane of host plant cells. Again these motifs were important for function because site-specific alteration to this motif abolished the virulence function of these genes.

Knowing that bacterial Avr proteins were hydrophilic, contained no signal peptide sequence and yet had motifs known to target the protein to internal areas of the host cell meant there must be some system responsible for transporting these effectors into the host cell. Insight into this mechanism came from observations that *Avr* gene function was lost when mutations in a group of genes known as *hrp* (hypersensitive response and pathogenicity) and *hrc* (hypersensitive response and conserved) genes were present (Collmer et al. 2000; Salmeron and Staskawicz 1993).

Together the *hrp* and *hrc* genes encode the type III secretion system (TTSS). This system forms a transmembrane pore that allows the release of Type III effectors essential for bacterial growth. Type III effectors include Avr proteins and hrp-dependent proteins (hop) which are proposed as important for release of bacteria to the leaf surface and nutrient acquisition (Chang et al. 2004). Genome sequencing of *P. syringae* pv. *tomato* DC3000 (Buell et al. 2003) and analysis of the Type III secretome indicates that there are approximately 40 Type III effectors (Guttmann et al. 2002; Petnicki-Ocwieja et al. 2002). However, the proportion which are Avr proteins, the amount of allelism that may exist for a given *Avr* gene and the number of *Avr* genes that may exist within a given pathogenic species remains unclear.

As the various extra- and intracellular locations of pathogen effectors were identified it became clear that corresponding R proteins could be found in the same cellular location. For example, the AvrPphB effector of *P. syringae* pv. *phaseolicola* contains a myristoylation motif as does the RPS5 R protein, which recognizes AvrPphB, and the PBS1 (AvrPphB susceptible 1) protein which is required by RPS5 for induction of the HR (Warren et al. 1998). In general, most of the bacterial effectors utilize the TTSS and

are therefore targeted to the inside of plant cells. Although the specific locations within the cell are not known for many, the nucleotide binding site-leucine-rich repeat (NBS-LRR) R proteins which recognize these effectors are also located inside plant cells. Some good examples of effector-R protein co-localization are found with fungal and oomycete pathogens and their hosts. These will be discussed in detail below.

Avirulence Gene Function II – Suppression of Host Defenses

Recently a number of studies have shed light on how pathogen effectors influence pathogen fitness and increase the pathogen's ability to infect host plants. Hauck et al. (2003) showed that suppression of cell wall defenses associated with basal defense is one such method by which effectors act. Expression of AvrPto in *Arabidopsis* suppressed callose deposition and papillae formation, enhancing the growth of TTSS mutants normally unable to infect. Microarray analysis revealed that expression of secreted cell wall defense proteins was also repressed.

Effectors from *P. syringae* have also been shown to suppress the HR. AvrPtoB is normally not recognized by Pto when expressed in tobacco. Using this information, Abramovitch et al. (2003) demonstrated that when AvrPtoB was transiently expressed in tobacco it could suppress the HR induced by either AvrPto-Pto or Avr9-Cf-9 interactions. Additionally, it inhibited heat and oxidative stress-induced cell death in yeast indicating it may act as a general suppressor of cell death.

Cell death inhibition has also been noted with effectors from the bean pathogen *P. syringae* pv. *phaseolicola*. It was initially noted that this pathogen suppressed the induction of defense genes undergoing HR (Jakobek et al. 1993). Later work demonstrated that the effectors VirPphA, AvrPphC and AvrPphF actually blocked the induction of the HR response (Jackson et al. 1999; Tsiamis et al. 2000). For example, AvrPphC blocked the HR triggered by AvrPphF in the cultivar 'Canadian Wonder.'

The best characterized pathogen effectors are the AvrRpt2 and AvrRpm1 proteins from *P. syringae*. Their interactions with their respective resistance genes, one another and components of the basal defense system demonstrate the complex and elegant mechanism(s) of virulence. This system will be described fully in the "Guard" Model section.

Fungal and Oomycete *Avr* Genes

The majority of information surrounding *Avr* genes is derived from bacterial pathogens, therefore, some comments specific to fungal and oomycete *Avr* genes are presented here. A limited number of *Avr* genes have been cloned from non-bacterial species. These genes include *Avr9* (Van Kan et al. 1991), *Avr4* (Joosten et al. 1994) and *Avr2* (Luderer et al. 2002) from *Cladosporium fulvum*, *Nip1* from *Rhynchosporium secalis* (Rohe et al. 1995), *AVR-Pita* from *Magnaporthe grisea* (Orbach et al. 2000), *AvrL567* from *Melampsora lini* (Dodds et al. 2004), *Avr1b-1* from *Phytophthora sojae* (Shan et al. 2004) and *ATR1*^{NaWsB} (Rehmany et al. 2005) and *ATR13* (Allen et al. 2004) from *Peronospora parasitica*.

Assigning a putative function to most of these genes based on sequence alone has been impossible due to the lack of homology with genes in existing databases. The lone exception is *AVR-Pita* which showed some similarity to zinc metaloproteases, but confirmation of protease activity has not been demonstrated (Orbach et al. 2000). However, a common feature of the proteins encoded by these *Avr* genes is that they are small and secreted. Although there is no secretory system in fungi and oomycetes analogous to the TTSS in bacteria, all Avr proteins, but one, contain a 15-23 amino acid N-terminal signal peptide which allows secretion of the protein from the pathogen. Once again the exception is the AVR-Pita effector. Although not analysed in all studies, it is likely that the signal peptide is cleaved by plant or fungal enzymes once secreted from the pathogen. For example, all of the effectors from *C. fulvum* are known to be processed from larger, signal bearing proteins to smaller, active forms lacking a signal peptide. Similarly, stronger HR are elicited with AvrL567 and ATR1^{NaWsB} effectors

lacking a signal peptide than those with one (Dodds et al. 2004; Rehmany et al. 2005). Interestingly, a N-terminal truncated form of AVR-Pita also caused a stronger HR than did the native protein, suggesting the effector may be secreted and processed by an unknown mechanism (Orbach et al. 2000).

A number of studies with fungal and oomycete *Avr* genes report, or indicate, co-localization of the pathogen effector and the corresponding plant resistance gene. For example, the Avr2, Avr4 and Avr9 effectors secreted from *C. fulvum* are located in the apoplast where this pathogen resides once inside the plant. The corresponding *Cf* resistance genes from tomato all consist of membrane-bound proteins with an extracellular LRR which would be able to interact with these effectors (Luderer et al. 2002). Intracellular recognition of the AvrL567 effector from *M. lini* implies that it is exported into in the cytoplasm of plant cells. *M. lini* produces haustoria that form intimate contact with the plant plasma membrane as part of the infection process. AvrL567 is expressed at highest levels in the haustoria and is presumably secreted from this structure into the plant cell (Dodds et al. 2004). The resistance genes at the *L* locus of flax that interact with AvrL567 alleles are cytoplasmic NBS-LRR proteins. Similar cytoplasmic co-localization of R-Avr partners are hypothesized for AVR-Pita-Pi-ta, ATR13-RPP13 and ATR1^{NaWsB}-RPP1.

While the role these genes play in disease development remains unknown for most, studies with Avr4 happened accidentally upon a function for this effector. To determine if there was a direct interaction between Avr4 and Cf-4, Westerink et al. (2002) attempted to find a high affinity binding site associated with proteins derived from tomato membranes infected with *C. fulvum*. Using ^{125}I-Avr4 and chemical crosslinking they identified a 75 kDa compound that showed all the characteristics of a receptor, that is, saturable, reversible and specific binding to Avr4. However, this compound was heat and Proteinase K insensitive suggesting it was not a protein, but more likely a polysaccharide. More surprising was that this compound was of fungal origin. The authors hypothesized the compound to be chitin and Avr4 protects the fungal cell wall

from cell wall degrading enzymes which would not only damage the hyphae, but could release elicitor-like molecules of chitin.

Subsequent analysis of Avr4 by Van den Burg et al. (2003) found the pattern of disulfide bonds and one area of sequence to be consistent with a chitin binding domain from invertebrate proteins. This was confirmed experimentally. They also noted that three of the four disulfide bonds were important for protein stability and that naturally occurring virulent mutants of *C. fulvum* showed disruption at two of these disulfide bonds (Cys to Tyr substitutions) resulting in increased sensitivity to protease, but maintenance of chitin binding activity. Protease sensitivity was also lessened when bound to chitin. These isoforms were never detected in the apoplast and thus never induced a *Cf-4*-mediated HR. The authors speculated that these Avr4 mutants maintain their virulence function by binding to chitin, but excess Avr4 secreted from the pathogen is quickly degraded by proteases in the apoplast and therefore never induces an HR. If true, this novel explanation of Avr4 function extends the varied activities of *Avr* genes.

The ability of Avr4-producing strains to overcome the corresponding *Cf-4 R* gene is a phenomenon noted with a number of other fungal and oomycete R-Avr interactions. Strains of *C. fulvum* virulent against the *Cf-2* gene harbour truncated Avr2 proteins, resulting from frame-shift mutations, or complete absence of the protein due to deletion of the *Avr2* gene (Luderer et al. 2002). No function has been assigned to Avr2 so it is unknown whether or not the truncated forms of Avr2 still retain a virulence function. Analysis of *M. grisea* strains virulent against the *Pi-ta* gene show a variety of point mutations, resulting in premature stop codons, and deletion/insertion events (including complete loss of the gene) associated with the *AVR-Pita* gene (Orbach et al. 2000). The ability of these pathogens to completely lose these genes and yet show no deleterious effects suggests that these genes may, like the *avrBs3* gene family in xanthomonads, be functionally redundant. In contrast, the *avrb1* gene from *P. sojae* shows only point mutations in virulent isolates which allow the pathogen to overcome the *Rps1b R* gene. After testing 40 isolates no deletion or other gross mutations were observed suggesting this gene may be unique and/or serve a more critical role in disease development, and

that the point mutations likely allow the maintenance of the Avr1b virulence function (Shan et al. 2004).

Fungal and oomycete *Avr* genes are also providing evidence for and new insights into theories of R-Avr interaction, such as the "guard" model and evolution models.

R-Avr Interactions - "Guard" Model

After the gene-for-gene hypothesis became widely accepted questions arose as to the nature of the R-Avr interaction at the molecular level. Many envisioned the resistance gene product acting as a receptor that bound to the matching avirulence ligand which in turn activated the host defense system (Gabriel and Rolfe 1990). The subsequent observation that the majority of plant resistance genes contain LRR (discussed later), which play an important role in protein-protein interactions (Kobe and Kajave 2001), provided further support for this theory.

The first such interaction was observed between the *avrPto* gene product from *P. syringae* pv. *tomato* and the *Pto* resistance gene product of tomato (Scofield et al. 1996; Tang et al. 1996). These groups demonstrated binding between the AvrPto and Pto proteins using the yeast two-hybrid system and, through mutation of the proteins, revealed a correlation between binding and disease resistance. Despite the fact that the *Pto* gene encoded a serine/threonine kinase and did not contain a LRR, these results seemed to validate the accepted model of R-Avr interactions and increased efforts were made to uncover other examples of binding between R proteins and effectors. However, only two other such interactions have been observed. The first was reported between AVR-Pita produced by *M. grisea* and the *Pi-ta* resistance gene product from rice (Jia et al. 2000). The *Pi-ta* gene was shown to be a member of the NBS-LRR class of *R* genes (Bryan et al. 2000), which was a significant finding for it was the first example of the hypothesized receptor-type model of *R* genes. More recently Deslandes et al. (2003) described a physical interaction bewteen RRS-1 from *Arabidopsis thaliana* and PopP2, a

Type III effector from *Ralstonia solanacearum*. RRS-1 was previously cloned and shown to be a NBS-LRR protein (Deslandes et al. 2002).

The lack of evidence suggesting direct binding between most R proteins and Avr proteins prompted the formation of new models. One such model that received much attention suggested the Avr protein is only recognized by R proteins once the Avr protein complexes with its virulence target. This concept was proposed by Van der Biezen and Jones (1998) to explain the need for Prf (*Pseudomonas* resistance and fenthion sensitivity), an NBS-LRR protein, in avrPto-Pto signalling (Salmeron et al. 1996) and became known as the "guard" model (Dangl and Jones 2001). They reasoned that Pto acts as part of the host basal defense system by interacting with other defense proteins (Zhou et al. 1997). These interactions are disrupted when avrPto binds to Pto which is consistent with the observation that avrPto is a virulence factor that acts by suppressing basal defenses (noted earlier) such as papillae formation, callose deposition and other cell wall associated changes (Hauck et al. 2003). Prf thus acts to guard Pto and activates plant defenses when it detects avrPto-Pto complexes, in effect, making *Prf* the true *R* gene.

An interesting insight into the guard model soon followed. Leister and Katagiri (2000), using immunoprecipitation experiments, demonstrated that the NBS-LRR R protein RPS2 from *Arabidopsis* formed a physical complex with its corresponding *P. syringae* Avr protein, AvrRpt2. However, they also found that it complexed with the structurally dissimilar Avr protein, AvrB, which is the partner of another *Arabidopsis* NBS-LRR R protein, RPM1 (resistance to *Pseudomonas syringae* pv. *maculicola* 1). They found that both RPS2-Avr protein complexes contain a common third protein (Figure 1.5).

Mackey et al. (2002) subsequently demonstrated that the protein RIN4 (RPM1 interacting 4) interacted with RPM1 and two *P. syringae* effectors recognized by this resistance gene, AvrB and AvrRpm1. RIN4 is thought to be a negative regulator of basal defense since basal defenses in the host were shown to be elevated in plants with reduced RIN4 expression. AvrB and AvrRpm1 induced hyperphosphorylation of RIN4,

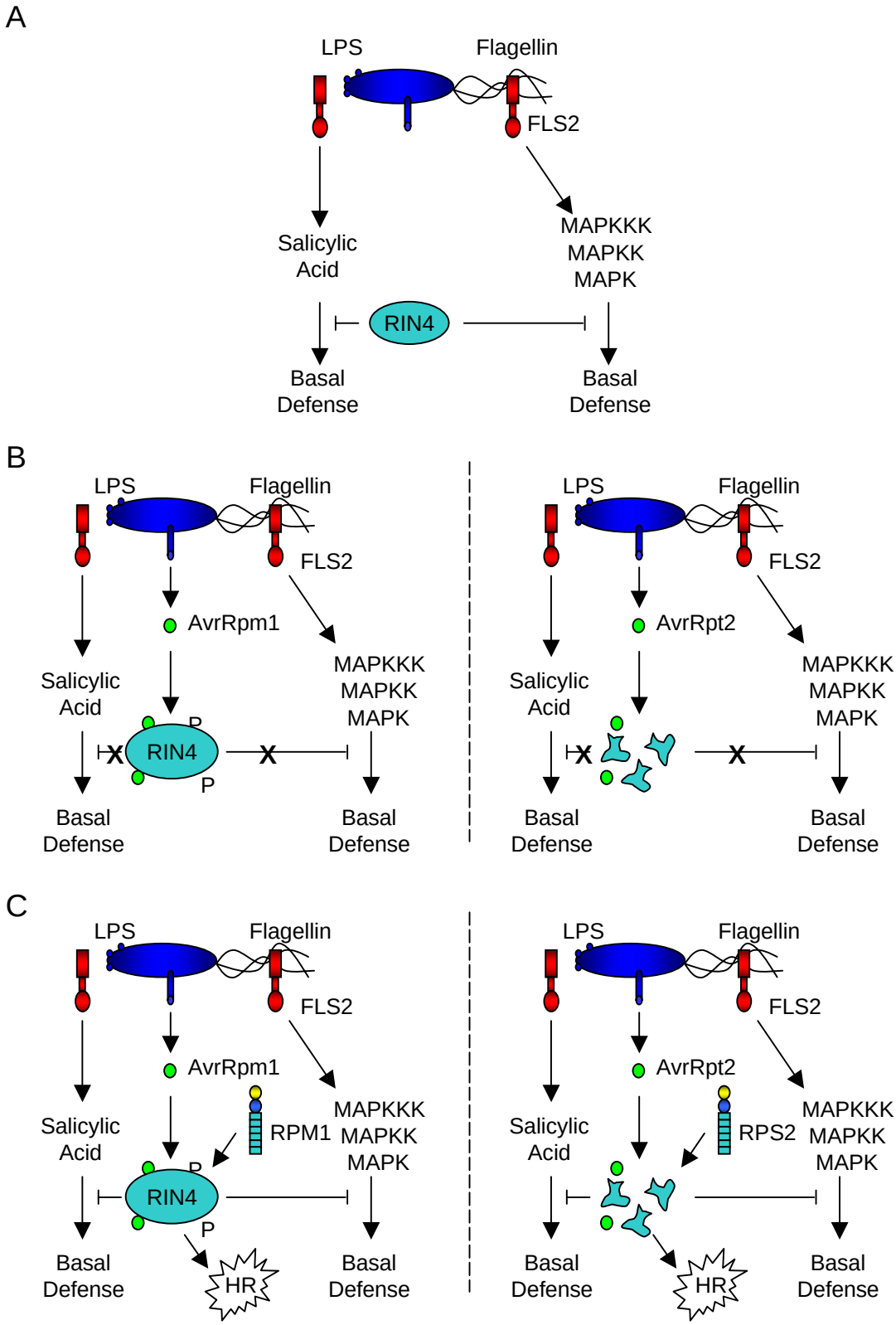

Figure 1.5. See caption on following page.

◄ Figure 1.5. Guard model for the AvrRpm1-RPM1 and AvrRpt2-RPS2 interactions. RIN4 is a negative regulator of basal defenses (A) and is the virulence target of both AvrRpm1, which phosphorylates, and AvrRpt2, which degrades, RIN4 (B). These changes to RIN4 disrupt basal defenses and allow the pathogen to infect host plants lacking the corresponding *R* genes. These changes to RIN4 are monitored by the resistance proteins RPM1 and RPS2 which induce the HR and prevent pathogen infection (C).

suppressing basal defenses. This again demonstrated a virulence function for pathogen effectors. RPM1-mediated defense responses depended on RIN4 so the authors proposed that RPM1 monitored RIN4 activity and upon phosphorylation, triggered a defense response.

RIN4 was later shown to physically interact with RPS2 and was essential for RPS2-mediated disease resistance against AvrRpt2 (Axtell and Staskawicz 2003; Mackey et al. 2003). AvrRpt2 caused the degradation of RIN4 (Axtell and Staskawicz 2003) providing an explanation for previous reports of AvrRpt2 suppression of basal defense (Chen et al. 2000). It was also shown that the *rin4* null mutant is seedling lethal, likely because this situation is similar to an infection by AvrRpt2 and triggers a defense response by RPM2. Therefore, RPS2 mutants should rescue the rin4 mutant and indeed such *rin4/rps2* double mutants are viable. These experiments showed that RPS2 monitors and acts upon the disappearance of RIN4 and does not interact directly with AvrRpt2.

These studies also helped explain the previous observation that AvrRpt2 interferes with RPM1-mediated resistance against AvrRpm1 (Ritter and Dangl 1996). When RPS2/RPM1 plants are infiltrated with AvrRpm1 and AvrRpt2, only RPS2-mediated resistance is exhibited. Based on the subsequent work, it is possible that RPM1 monitoring of RIN4 phosphorylation is inhibited when AvrRpt2 degrades RIN4.

Another variation on the guard model comes from the apparent direct interaction between RRS-1 and PopP2 mentioned earlier. The RRS-1 gene encodes a NLS and a C-terminal WRKY (tryptophan-arginine-lysine-tyrosine) domain. WRKY domains are commonly found in plant transcription factors that bind to W-box domains in the promoter regions of certain genes, including PR genes. This led the authors to conclude

that RRS-1 functioned by combining direct recognition of PopP2 with transcriptional activation of plant defense genes. However, flourescent tagging of RRS-1 showed it was complexed with PopP2 in the cytoplasm indicating the NLS signal was non-functional. The combination of a non-functional NLS and a WRKY domain have led to the suggestion that the WRKY domain may act as bait for PopP2. PopP2 contains a functional NLS and its virulence function may be to interfere with WRKY transcription factors that induce plant defense-related gene expression. Therefore, RRS-1 may attempt to misdirect PopP2 by providing its own WRKY domain to which PopP2 could bind. If so, it would appear that RRS-1 is still guarding certain proteins by incorporating some of their structure into itself.

The Avr2-Cf-2 interaction also lends support to the guard model. Cf-2 function requires Rcr3 (Dixon et al. 2000), a secreted cysteine protease (Kruger et al. 2002) not required by other *Cf* resistance genes. Luderer et al. (2002) noted a correlation between the compromised HR produced in several rcr3 mutant lines in response to Avr2 alone and strains producing Avr2. This led to the hypothesis that Rcr3 is the virulence target of Avr2. Rooney et al. (2005) showed that Avr2 binds to and inhibits the protease activity of Rcr3 and that initiation of the Cf-2-mediated HR required both Rcr3 and Avr2. They proposed that Cf-2 monitors a conformational change to Rcr3 when bound by Avr2 which leads to the HR. It was also suggested that the protease activity of Rcr3 was an antimicrobial plant defense and thus its inhibition was the virulence function of Avr2.

One other well-defined interaction involving the *Arabidopsis* RPS5 resistance protein and the *P. syringae* AvrPphB effector is also consistent with the guard model. RPS5 is a cytoplasmic NBS-LRR protein (Warren et al. 1998) that specifically requires PBS1 to induce the HR in response to AvrPphB (Swiderski and Innes 2001). PBS1 is a serine/threonine kinase which shows homology to the tomato Pti1 (Pto interacting 1) kinase at the N-terminal. Over-expression of Pti1 is known to enhance Pto-mediated HR induced by AvrPto, suggesting that Pti1 plays a role in Pto-mediated resistance (Zhou et al. 1995). Swiderski and Innes (2001) proposed PBS1 may also regulate defense responses although no experimental evidence is yet available. AvrPphB is a cysteine

protease (Shao et al. 2002) that is able to bind and cleave PBS1 in the activation segment of the kinase domain (Shao et al. 2003). Shao et al. (2003) also demonstrated that a PBS1 mutant lacking kinase activity, but cleaved by AvrPphB, did not induce a HR. Similarly, another PBS1 mutant lacking the cleavage site, but retaining kinase activity could not induce a HR. The authors proposed that PBS1 is cleaved by AvrPphB and that one of the cleavage products complexed with AvrPphB is recognized and activates RPS5, leading to a HR. They also suggest that the cleavage product must be autophosphorylated since kinase activity was shown to be required for HR induction.

An increasing body of work conforms to the guard model. It appears that protease activity in pathogen effectors, as observed with AvrRpt2, AvrPphB and potentially AVR-Pita, may be a common strategy to inactivate key plant regulatory defense proteins. Similarly, conformational change in virulence target proteins appears to be the mechanism by which most resistance proteins monitor pathogen activity. However, the chitin-binding function of Avr4 and the "bait" domain on RRS-1 offer insights into the potentially diverse strategies employed by both plants and pathogens.

Studies with RIN4 showed that one protein could be the target of multiple effectors and *R* genes. It may be that the key role RIN4 plays in maintaining basal defense lends it, and other such important proteins (possibly PBS1), to becoming the target of common pathogen strategies to overcome the plant host. By producing a smaller number of *R* genes to monitor key virulence targets, plants would not have to use the costly strategy of one *R* gene per effector. Indeed, after extensive manual re-annotation of the *Arabidopsis* genome sequence, Meyers et al. (2003) have only detected 149 NBS-LRR genes, and given the large number of potential pathogens, and thus effector proteins, this may be the strategy plants have adopted.

Resistance Genes

Evolution

A brief mention of plant *R* gene evolution models as they pertain to plant-pathogen interactions is warranted since there are implications to *Avr* gene function and the gene-for-gene concept.

Sequence analysis of *R* genes reveals a large amount of genetic variability, expressed as multiple alleles, gene clusters and multiple loci, indicating a long co-evolution between host and pathogen. The classic "arms race" model for *R* gene evolution describes a situation in which a pathogen produces a new virulence factor which is eventually recognised by a novel *R* gene that quickly becomes prevalent in the population. This is followed by successive and continual cycles of novel pathogen virulence factors overcoming host defenses and corresponding host *R* genes created to compensate.

Evidence supporting this adaptive evolution comes from sequence comparisons of *R* gene paralogues on the same chromosome. Studies have focused on the NBS-LRR family because their only recognized function is in disease resistance. More specifically, the LRR has been the focus of attention because of its role in avirulence factor recognition specificity.

Adaptive evolution is assessed by looking at non-synonymous amino acid substitution rates versus synonymous substitutions in the same gene, with the assumption that more non-synonymous amino acid substitutions are indicative of positive selection pressure. Studies in tomato (Parniske et al. 1997), rice (Wang et al. 1998) and *Arabidopsis* (Noel et al. 1999) all found the rate of non-synonymous substitution to be nearly twice that of synonymous substitution, consistent with adaptive evolution.

The arms race theory also implies that, with high disease pressure, old *R* genes will be replaced quickly by new ones resulting in "young" *R* genes and monomorphic *R* gene loci. However, there are several lines of evidence that dispute this reasoning. Studies of the *RPM1* and *Pto* genes in *Arabidopsis* and tomato, respectively, show that these genes existed prior to speciation (Riely and Martin 2001; Stahl et al. 1999). Also, the *Cf-2* homologues isolated from wild populations of tomato showed a high level of variation (Caicedo and Schaal 2004), as does the *RPP13* (resistance to *Peronospora parasitica* 13) locus analysed in 24 accessions of *Arabidopsis* (Rose et al. 2004). Finally, studies have shown that the highest levels of polymorphism are maintained in geographical regions with the greatest disease pressure (Leonard 1997).

This indicates that *R* genes are more likely maintained in a polymorphic state in a population through "balancing selection." Thus, an *R* gene will become more frequent in a population as a result of its selective advantage and decline in frequency as the corresponding pathogen causes less disease pressure (Van der Hoorn et al. 2002). Important to this balance is the cost of virulence to the pathogen and the cost of resistance to the host.

As mentioned, many avirulence genes have virulence functions that contribute to a pathogen's fitness. The presence of a virulence function is critical to balanced polymorphism of *R* genes. If an avirulence factor had no virulence function, the selection pressure on the pathogen imposed by a plant population with the matching *R* gene would result in selection for loss of that avirulence gene. We would therefore not see both *R* and *S* allele maintenance within plant populations and *Avr* genes in pathogens (Van der Hoorn et al. 2001). From the pathogen's perspective, a balance must be struck between the virulence function associated with the *Avr* gene and any fitness cost incurred by mutation of this gene. Thus, with respect to a specific R-Avr interaction, mutation to a non-functional *Avr* gene (ie. to virulence) will increase pathogen fitness if the corresponding *R* gene is present, but may hinder pathogen fitness in the absence of the *R* gene (Van der Plank 1968).

Several recent studies of fungal and oomycete *Avr* genes have noted that high levels of polymorphism exist corresponding with the large number of alleles present in their matching *R* genes. For example, Allen et al. (2004) cloned and analyzed the *ATR13* avirulence gene from *P. parasitica*, which matches the highly divergent *RPP13* resistance gene from *Arabidopsis* mentioned above. They identified five different alleles from six isolates and noted 26 non-synonymous and only two synonymous polymorphisms, primarily in the C-terminal end of the protein. The alleles elicited differential HR in *Arabidopsis* lines carrying different alleles of *RPP13*. The excessive number of non-synonymous substitutions indicates that a high level of polymorphism is maintained in this gene, consistent with the concept of balanced selection. The diversity in the C-terminal of ATR13 and the LRR of RPP13 also indicates that these regions are likely important for allele-for-allele interaction.

Analysis of the *AvrL567* gene from *M. lini* also supports the balancing selection theory. Two different cloned alleles showed 30 nucleotide changes within the 450 bp coding region, in comparison, only 25 nucleotide changes were found in the 7000 bp of flanking sequence. Also, 27 of the 30 changes gave rise to non-synonymous amino acid substitutions (Dodds et al. 2004). The two alleles were also differentially recognized by the *L5*, *L6* and *L7* resistance genes. Similar observations have been reported for the ATR^{NdWsB} and *Avr1b-1* genes from *P. parasitica* (Rehmany et al. 2005) and *P. sojae* (Shan et al. 2004), respectively.

Van der Hoorn et al. (2002) suggest that *R* gene dynamics in plant populations likely include elements of both balancing selection and an arms race (Figure 1.6). *R* gene creation is random and constant according to the birth-death model (Michelmore and Meyers 1998) and in many cases *R* genes will have no function and will be lost over time. However, some will by chance recognize some pathogen factor and be retained. This will result in a selective advantage and the *R* gene's frequency in the population will increase. The *R* gene may become extinct if the pathogen can mutate the corresponding avirulence factor without causing a loss in virulence function. If this is not possible the *R* gene will be maintained in the population at a frequency

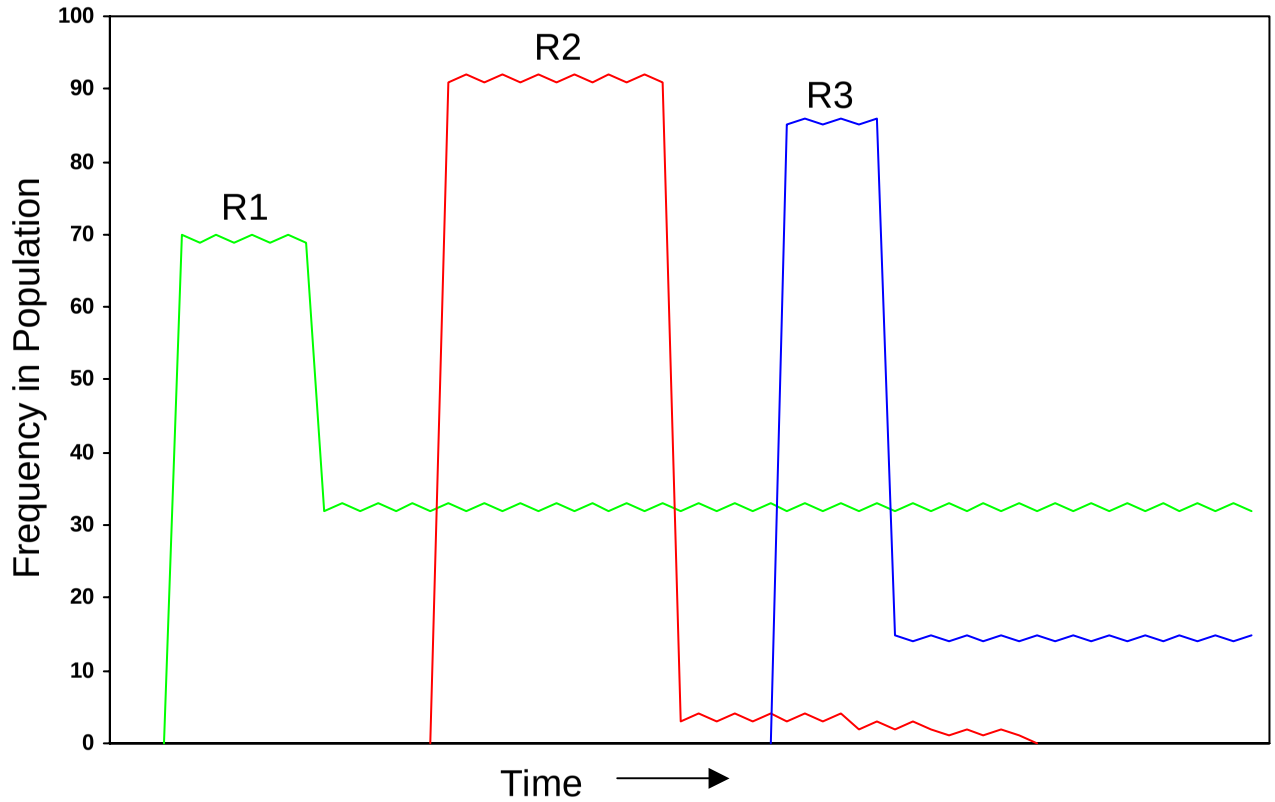

Figure 1.6. *R* gene frequency in plant populations as described by balancing selection. The steady-state frequency of an *R* gene is proportional to the importance of the corresponding effector on pathogen fitness. R1 and R3 are maintained because the effectors they recognize can not be completely deleted, or modified significantly, without significant fitness penalty to the pathogen. R2 is eventually lost because the pathogen can delete or modify the corresponding effector with no effect on fitness. Adapted from Van der Hoorn et al. (2002).

corresponding to the importance of the matching avirulence factor. It should be noted that this model reflects the dynamics of *R* genes in natural populations. This differs from the typical boom-bust cycle observed with the total resistance required for monocultures. In these circumstances, pathogen strains can overcome *R* genes even with a fitness cost because there will be no competing strains present.

The balancing selection model also provides support for the guard model hypothesis. In a situation where two separate *R* genes which target the same avirulence factor evolve, the first directly interacting with the avirulence factor while the second detects a modification to the virulence target (Figure 1.7), it would be much simpler for a pathogen to overcome the first *R* gene by altering the binding site recognized by the *R* gene. In contrast, such structural changes in the avirulence factor would not affect the functioning of the second *R* gene. From a plant's perspective, this provides a more durable type of resistance.

Classes

Since the early 1990s over 40 *R* genes have been cloned, conferring resistance to bacterial, fungal, viral, oomycete and even insect and nematode pathogens. Although the range of pathogens and potential avirulence factors is large, there are only five classes of *R* genes (Figure 1.2).

The largest classes of *R* genes are defined by their NBS-LRR regions. This group can be subdivided further based on N-terminal structure. One group shows N-terminal homology to the intracellular signalling domains of the *Drosophilia* Toll and mammalian interleukin (IL)-1 receptors (TIR-NBS-LRR), while the second subgroup contains a coiled-coil domain at the N-terminal (CC-NBS-LRR). At present, the only known function for this family of genes is in disease resistance.

The other four classes of *R* genes are represented by only one or a few genes. Since these classes have roles in plant development and other cellular functions, their role in

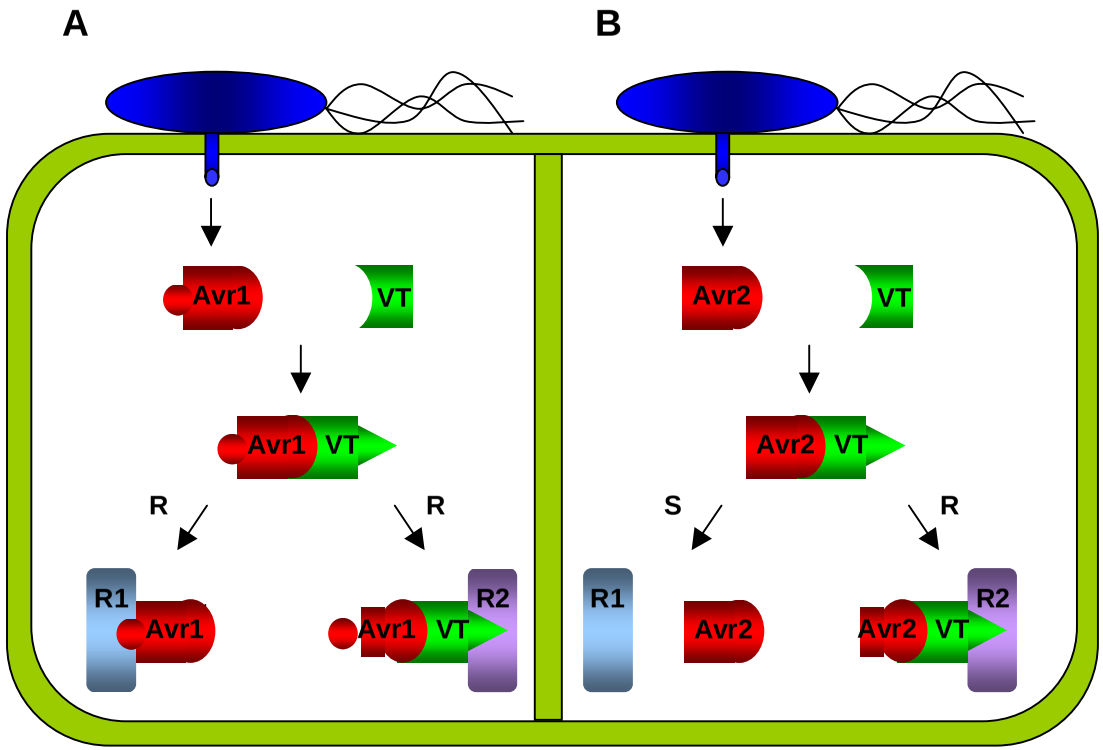

Figure 1.7. Balancing selection supports the guard hypothesis. (A) Direct recognition of the effector by the resistance protein, or indirect recognition by monitoring changes to the virulence target of the effector both lead to resistance. (B) Changes in the effector structure circumvent direct recognition by the resistance protein and lead to susceptibility. However, changes in structure do not affect indirect monitoring of the virulence target and resistance is maintained. Adapted from Van der Hoorn et al. (2002).

plant defense may be exceptional. One such class is represented solely by the *Pto* gene from tomato which encodes an intracellular serine/threonine kinase. The *Xa21* gene from rice is the only representative of a related class of genes which combine an intracellular serine/threonine kinase to a transmembrane region and an extracellular LRR. The *Cf* family of *R* genes from tomato represents the last class of genes which consist of a TM region attached to an extracellular LRR.

There is also a collection of *R* genes that encompass a structurally diverse group that do not fit within the other classes. The *RPW8* gene from *Arabidopsis* confers wide-spectrum resistance to powdery mildew and contains a TM connected to an intracellular CC domain (Xiao et al. 2001). The barley *Mlo* gene, which also provides broad spectrum resistance to powdery mildew, is a membrane bound protein (Buschges et al. 1997). The *Rpg1* gene from barley, controlling stem rust resistance, has two tandem kinase domains and a potential transmembrane region (Brueggeman et al. 2002).

The LRR is an important region for protein-protein interactions. It consists of a short stretch of amino acids with leucine residues repeated at every second or third position to form a flexible, solvent exposed beta-sheet. The importance of this region for binding to pathogen effectors has been demonstrated for the rice *Pi-ta* gene with its cognate effector, AVR-Pita from the rice blast pathogen (Jia et al. 2000). Although only a few studies have demonstrated such direct binding, other studies show that the LRR of *R* proteins bind to host factors which are the target of pathogen virulence factors. For example, the LRR of the *RPM1 R* protein binds to the *Arabidopsis* RIN4 protein which is the target of three pathogen effectors, AvrB, AvrPpt2 and AvrRpm1 (Leister and Katagiri 2000; Mackey et al. 2002). Several studies have demonstrated that mutation of key residues in the LRR region destroys binding ability, but also that the region is tolerant to a large number of substitutions (Axtell et al. 2001; Tornero et al. 2002). Mondragon-Palomino et al. (2002) have shown that this region is under evolutionary selection and is key to developing *R* gene variability in order to adapt against new pathogen factors.

The NBS region is found in other protein families like ATPases and G proteins which suggest a role in nucleotide binding or hydrolysis. Van der Biezen and Jones (1998) noted the similarity of the NBS region in *R* genes to regions in the APAF-1 and CED-4 proteins which are important regulators of PCD in animals. This would not be inconsistent with a similar function in plants, namely the HR.

The CC domain consists of a repeated heptad sequence intermixed with hydrophobic amino acids. The leucine zipper is an example of a CC structure. There are typically two or more alpha helices which interact to form a supercoil. Typically CC domains are important in protein-protein interactions including oligomerization. Studies with *R* genes suggest this may be important for signalling rather than recognition. This is based on the observation that in *Arabidopsis*, the TIR-NBS-LRR *R* genes require the downstream protein EDS1 while the CC-NBS-LRR *R* genes require NDR1 and PBS2 for expression of defense responses. Thus, two apparently exclusive, but parallel defense-signalling pathways are employed by these two groups of *R* genes distinguishable only by their TIR or CC domains (Van der Biezen et al. 2002).

The TIR region is also believed to be involved in signalling because of its similarity to the cytoplasmic domain of Toll and Interleukin-1. The separate defense signalling pathways mentioned above for the CC domain also indicate a role in signalling. Luck et al. (2000) showed that the TIR of the flax *L* gene was important in determining pathogen recognition.

The kinase activity of the Pto gene has been studied extensively and provides insight into how such domains function in resistance. Autophosphorylation of Pto has been demonstrated as necessary for its function (Sessa et al. 2000), as well, Pto phosphorylates a group of proteins known as Pti proteins. Zhou et al. (1995) showed that Pto phosphorylates Pti1, also a serine/threonine kinase, which is involved in signalling and leads to induction of the HR. Pti4, Pti5 and Pti6 are transcription factors that bind to the GCC box which is a common *cis*-element found in the promoter of many PR genes (Gu et al. 2000; Zhou et al. 1997). Phosphorylation of Pti4 by Pto enhances

binding to the GCC box and overexpression of this gene leads to enhanced expression of PR genes.

Host-Selective Toxins

Approximately 20 HSTs have been identified (Walton 1996). They represent a varied group of compounds produced by phytopathogens that are only active on the pathogen host. Their production is essential to elicit disease, thus they are regarded as pathogenicity factors, as opposed to virulence factors that affect the level of disease caused. HSTs are produced in only a few species of fungi, the best characterized being from species of *Cochliobolus*, *Alternaria* and *Pyrenophora*. HSTs provide an interesting comparison to Avr factors and the two groups have typically been described as opposites in terms of how they mediate plant-pathogen interactions. HSTs conform to the 'toxin' model, where compatibility is the basis of specificity between a particular host line and pathogen race (Figure 1.4). This is in contrast to the 'gene-for-gene' model where incompatibility forms the basis of specificity. However, HSTs may be more similar to Avr factors than originally thought.

HSTs are typified by several common characteristics: 1) they tend to be low molecular weight secreted compounds, 2) they are produced by pathogens that are necrotrophic at some point in their lifecycle, 3) strains lacking the HST or carrying mutated isoforms are unable to cause disease, 4) sensitivity to the toxin and susceptibility to the pathogen are conferred by single, dominant genes that co-segregate, and 5) induction of PCD appears to be a common mode of action.

Victoria blight of oats is a well known example of a disease caused by an HST-producing pathogen and typifies many of the features of HST-mediated interactions. The disease is caused by strains of *Cochliobolus victoriae* which produce the HST victorin (Wolpert et al. 1985). Victorin is a chlorinated, cyclized pentapeptide and

strains unable to produce victorin are unable to cause disease on oats (Wolpert et al. 1985). Sensitivity to the toxin is conferred by the *Vb* locus, with the fully recessive genotype being resistant to victorin producing strains.

Initial reports of Victoria blight noted the disease was only found on Victoria-type oat varieties which contained the *Pc2* crown rust resistance gene (Meehan and Murphy 1946). Unsuccessful attempts to break this linkage lead to the hypothesis that *Pc2* and *Vb* may be the same gene (Luke et al. 1966; Mayama et al. 1995). If true, this would present an interesting explanation for a number of phenotypic responses typical of PCD, such as DNA laddering, heterochromatin condensation and mitochondrial disruption, that have been observed with victorin toxicity (Tada et al. 2001; Yao et al. 2001). As well, basal defense-type reactions such as PR gene synthesis (Tada et al. 2005) and callose synthesis (Walton and Earle 1985) have also been observed in response to victorin.

To elucidate the victorin mode of action, Wolpert et al. (1994) radiolabelled victorin and found it bound to the glycine decarboxylase complex (GDC) located in the mitochondria and strongly inhibited its activity. However, in order to enter the mitochondria a permeability transition must first occur which suggested that victorin interacted with the *Vb* gene product prior to this transition, and it is therefore possible that GDC inhibition is not the main cause of PCD. Recently, Tada et al. (2005) demonstrated that flourescene-labelled victorin does not cross the cell membrane before activation of PCD, suggesting that victorin is recognized on the cell membrane surface.

Since the HR response is mediated by *R* genes and is a form of PCD, the idea that victorin is able to subvert *Pc2* function by inducing it to produce a premature HR (ie. in the absence of its corresponding effector) is appealing. Tada et al. (2005) also suggested that any activated defenses such as PR expression and callose deposition would be slow in comparison to PCD and thus would not be able to mount an effective defense. Initiation of PCD would be beneficial to this necrotroph because these pathogens are only able to colonize dead host cells to acquire nutrients.

The T-toxin produced by *C. heterostrophus* provides another example where PCD is induced. T-toxin is a mixture of small (C_{35}-C_{41}) linear polyketols which incite Southern corn leaf blight in maize lines carrying Texas cytoplasmic male sterility (*T-cms*). It is able to disrupt mitochondrial function, as observed by the uncoupling of oxidative phosphorylation and the leakage of small molecules from the mitochondria (Levings et al. 1995). Analysis of mitochondrial DNA from *T-cms* germplasm showed that it contained a unique gene, *T-urf13* (Dewey et al. 1986), which encoded a small peptide that formed a mitochondrial membrane localized tetramer (Dewey et al. 1987). T-toxin binds to this tetramer and induces a conformational change resulting in the formation of a pore (Levings et al. 1995). Loss of mitochondrial function would disrupt the ability to regulate PCD and induce premature cell death, again to the benefit of this necrotrophic pathogen.

These examples highlight pathogens which produce a single HST that allows the pathogen to infect a previously inaccessible host. Such toxins are considered to increase the host range of the pathogen. In contrast, *P. tritici-repentis* produces three HSTs effective against the same host species (wheat) which mediate compatibility with different host lines in a manner reminiscent of the gene-for-gene model. Also, two of the three HSTs are larger peptides (Ptr ToxA is 178 amino acids and Ptr ToxB is 87 amino acids) containing 22 and 23 amino acid signal peptides, respectively, similar to the effectors produced by fungal pathogens mentioned earlier (Balance et al. 1989; Ciuffetti et al. 1997; Martinez et al. 2001; Strelkov 2002).

The three Ptr toxins (Ptr ToxA, B and C) allow the differentiation of eight races based on the variable expression of necrosis or chlorosis symptoms on three differential wheat lines (Strelkov and Lamari 2003). As with other HSTs, the presence of a dominant, fully functional toxin gene is required to elicit the unique necrosis or chlorosis symptoms associated with each toxin, and single, dominant and independently inherited genes are responsible for sensitivity to each of the toxins. However, there is some debate whether these HSTs are pathogenicity factors like other HSTs, or are simply

virulence factors like *Avr* gene products. Wheat lines rendered insensitive to Ptr ToxA by EMS were not necessarily resistant to race 1 (Ptr ToxA$^+$ and Ptr ToxC$^+$) isolates (Friesen et al. 2002) and showed incomplete resistance to race 2 (Ptr ToxA$^+$) (Friesen et al. 2003). If we assume that the dominant sensitivity genes encode toxin receptors for each of the toxins, then susceptibility to race 1 isolates could be explained by the presence of a Ptr Tox C receptor. Susceptibility to race 2 could be explained by the presence of unidentified toxins. For example, Lamari et al. (1995) showed that some race 5 isolates (Ptr ToxA$^-$) are able to induce necrosis on several durum lines insensitive to Ptr ToxA. It appears that the relationship between *P. tritici-repentis* and wheat is more complicated than other HST-producing pathogens and their respective hosts, yet not as complex as R-Avr type interactions. It possibly represents an intermediate between the two systems.

The ability of a pathogen to expand its host range is generally thought to occur by the acquisition of HSTs through the phenomenon of horizontal gene transfer. This concept has been used to explain the presence of T-toxin in *C. heterostophus* (Yang et al. 1996), victorin in *C. victoriae* (Rosewich and Kistler 2000), HC toxin in *C. carbonum* race 1 (Ahn and Walton 1996) and AK toxin in the *Alternaria alternata* pear pathogen (Tanaka et al. 1999). While there is no data regarding when the various HST-producing pathogens moved to their "new" hosts, it is tempting to speculate that HSTs represent the earliest stages (evolutionarily) of the gene-for-gene interaction model. The single HST producing pathogens may have taken the first step that will lead to the development of a host *R* gene in response to the HST, changing the HST from a pathogenicity factor to a virulence factor and requiring the pathogen to develop further virulence factors.

Perhaps the multiple HSTs of *P. tritici-repentis* represent further advancement in the transition to a gene-for-gene model. It is interesting to note that the three Ptr toxins exist in races found around the world (Strelkov and Lamari 2003), indicating that these genes have existed within the *P. tritici-repentis* genome for a substantial period of time, likely since *P. tritici-repentis* became a pathogen of wheat. This would suggest that some co-evolution between the two genomes has occurred, and perhaps the multiple Ptr ToxB

homologues are indicative of selection pressure. In this light, the Ptr ToxA gene may represent a newer acquisition than Ptr ToxB which has not yet had time to change.

Other features of HSTs show similarities to Avr proteins. The Ptr ToxB protein is similar to the small secreted Avr2, Avr4 and Avr9 of *C. fulvum* and Nip1 protein from *R. secalis*. The Ptr ToxA protein has recently been shown to be imported into wheat cells where it is able to induce PCD (Manning and Ciufetti 2005). This import into plant cytoplasm is similar to that described for AvrL567 and ATR13 of *M. lini* and *P. parasitica*. Elucidation of the victorin mode of action indicates that, like Avr proteins, disruption of host defenses is a common theme. In light of these observations it is tempting to imagine gene-for-gene relationships developing with these toxins.

Implications of R-Avr Knowledge on Durable Resistance

Durable resistance has been documented for a number of plant diseases. For example, the barley *Rpg1* gene has effectively controlled stem rust for over 60 years (Brueggeman et al. 2002), while the wheat *Lr34* gene provided leaf rust resistance for > 30 years (Kolmer 1996) and the rice *Xa4* gene was effective against *X. oryzae* pv. *oryzae* for about 10 years in the Philippines (Mew 1987). However, the reasons for such durability were unknown, therefore, predicting the durability of *R* genes before deployment into varieties was impossible. However, it was evident that durability was a rare event.

The study of *R* genes, *Avr* genes and their interactions has begun to shed light onto the phenomenon of durable resistance. This work has not only revealed how *R* genes are overcome (by mutation of the corresponding *Avr* genes which abolishes recognition by the *R* gene), but has also provided theories explaining the nature of *R* gene durability. The hypothesis, and tentative support, provided by Vera Cruz et al. (2000) that *R* gene durability is directly related to the importance that the corresponding *Avr* gene has on pathogen fitness is a simple and attractive explanation. However, a number of

observations demonstrate that the relationship between R-Avr pairs is complex and underscore the elusive nature of durable resistance.

Work with fungal pathogens demonstrates allelic variation in both pathogen avirulence and plant resistance genes exists and that compatible pairs must be present to induce the HR. If a virulence target is an essential component of the R-Avr interaction, then isoforms of the virulence target may also exist and affect recognition by the resistance protein. Such an occurrence could explain the existence of digenic resistance, that is, a cross between two susceptible lines resulting in a resistant line (Buell and Somerville 1997). At present there are no reports investigating polymorphism in virulence target proteins. However, there are a number of examples demonstrating that R proteins can recognize their corresponding effector in distantly related species. For example, the Cf-4-Avr4 interaction will induce the HR in *L. sativa* (Van der Hoorn et al. 2000), Cf-9 will recognize Avr9 and induce the HR in *Brassica napus* (Hennin et al. 2001) and L6-AvrL567 produces the HR in *Nicotiana tabacum* (Dodds et al. 2004). These examples indicate that a high level of conservation of virulence target proteins does exist and that allelic variation may not be a concern, especially within a species. There are examples with *Bs2* (Tai et al. 1999) and *Cf-9* (Van der Hoorn et al. 2000) where no induction of the HR was observed when presented with their corresponding effectors, but only at the species level.

Although many avirulence genes demonstrate some function associated with pathogen fitness and virulence, there are cases where no contribution to fitness can be attributed to an avirulence gene. The *AVR-Pita* gene from *M. grisea* (Orbach et al. 2000) and the *avrXa10* gene from *X. oryzae* pv. *oryzae* (Bai et al. 2000) have already been mentioned. In these cases, questions remain as to their purpose and why they have not been eliminated from the gene pool through selection. Functional redundancy is a potential explanation. Some avirulence genes, such as the *avrBs3* family in *X. campestris* pv. *malvaraceum*, are present in multiple copies which contribute to virulence in an additive and redundant manner (Yang et al. 1996). This may mean that the loss of any one gene from this family could be compensated for by other members of the family already

present, or those which may be acquired by horizontal gene transfer from other strains. If so, *R* genes deployed against any individual *Avr* gene belonging to such families would not be expected to be durable, thus assessing functional redundancy would be an important consideration.

Finally, it is also interesting to note that the genetic background in which an *Avr* gene resides can affect its function. For example, mutation of the *avrPto* gene decreased the lesion size and multiplication rate in strain T1, but showed no such effect in strain DC3000 (Shan et al. 2000). In contrast, the *pthA* gene from *Xanthomonas citri*, which is required for virulence when infecting citrus plants, is able to confer its function when transferred into other xanthomonads (Swarup et al. 1992). Clearly, determining the stability of *Avr* gene fitness functions would be an important consideration when determining *R* gene durability.

There is limited evidence that *R* genes can decrease plant fitness in the absence of pathogen pressure. Tao et al. (2000) showed that overexpression of *Rps2* in *Arabidopsis* is lethal, while *Rpm1* has been lost from different accessions of *Arabidopsis* on numerous occasions indicating that it might also carry a fitness cost (Stahl et al. 1999). If true, then the concept of pyramiding *R* genes as a strategy to achieve durable resistance may need to be re-evaluated since the presence of several *R* genes could significantly affect plant growth. An alternative would be to deploy several *R* genes in a common genetic background for release as a mixture. The benefits of such mixtures were demonstrated by Zhu et al. (2000) when a rice blast sensitive rice line was grown with a resistant line and 25-fold less disease was observed on the susceptible line in comparison to when grown alone. These observations are in line with the high levels of *R* gene polymorphism reported in natural plant populations where high disease pressure, but no epidemics, exists (Leonard 1997). Mixtures of *R* genes reduce the pressure imposed on the pathogen to mutate and could lead to different consequences. For example, defeat of any one *R* gene limits the new race to only a fraction of the plant population, thus decreasing the rate of epidemic spread. Also, maintenance of more pathogen races, a portion of which will be avirulent on the various plant lines, may

activate SAR and reduce susceptibility to the virulent races. In contrast, the selection pressure imposed on pathogens by pyramided *R* genes is essentially the same as using single *R* genes, resulting in the typical boom-bust cycle.

Chapter 2: Identification of Molecular Markers Linked to a *Pyrenophora teres* Avirulence Gene

Abstract

This study investigated the genetic control of avirulence in the net blotch pathogen, *Pyrenophora teres*. To establish an appropriate study system, a collection of ten net form (*P. teres* f. *teres*) and spot form (*P. teres* f. *maculata*) isolates were evaluated on a set of eight differential barley lines to identify two isolates with differential virulence on an individual host line. WRS 1906, exhibiting low virulence on the cultivar 'Heartland,' and WRS 1607, exhibiting high virulence, were mated and 67 progeny were isolated and phenotyped for reaction on Heartland. The population segregated in a 1:1 ratio, 34 avirulent to 33 virulent ($\chi^2 = 0.0$, $P = 1.0$), indicating single gene control of WRS 1906 avirulence on Heartland. Bulked segregant analysis was used to identify six amplified fragment length polymorphism (AFLP) markers closely linked to the avirulence gene ($Avr_{Heartland}$). This work provides evidence that the *P. teres*-barley pathosystem conforms to the gene-for-gene model and represents an initial step towards map-based cloning of this gene.

Introduction

Pyrenophora teres Drechs. (anamorph: *Drechslera teres* (Sacc.) Shoem.) is the causal agent of barley net blotch. Net blotch is a common, persistent disease found in all barley producing regions of the world and is therefore of economic concern to the barley industry. Financial losses to this foliar pathogen result from both yield reduction, ranging from 15% to 35% (Khan 1987; Martin 1985; Steffenson et al. 1991), and diminished grain quality (Mathre 1997).

Studies have reported a large number of *P. teres* pathotypes. For example, Tekauz (1990) identified 45 net form (*P. teres* f. *teres*) and 20 spot form (*P. teres* f. *maculata*) pathotypes using a set of nine and 12 differentials, respectively. Similarly extensive pathotype variability has been described from California (Steffenson and Webster 1992) and the former USSR (Afanasenko and Levitin 1979). Within the barley genome, loci controlling net blotch resistance have been identified on every chromosome. While several studies have located quantitative trait loci (QTL) associated with resistance (Raman et al. 2003; Steffenson et al. 1996), a number of single, major genes controlling resistance have been identified (Afanasenko et al. 1995) with several mapped (Graner et al. 1996; Manninen et al. 2000; Williams et al. 1999).

The numerous *P. teres* pathotypes and barley resistance genes suggest this pathosystem likely conforms to the classic gene-for-gene model (Flor 1955). Much progress in understanding the molecular basis of this model has been made via the cloning of many plant resistance (*R*) genes and their cognate pathogen avirulence (*Avr*) genes. One significant aspect of this work has been the discovery that pathogen *Avr* gene products (effectors) contribute to a pathogen's ability to infect its host by suppressing various host defenses. For example, both AvrRpt2 and AvrRpm1 from *Pseudomonas syringae* pv. *tomato* can disrupt basal defenses, such as callose deposition, by altering RIN4, a regulator of pathogen associated molecular pattern (PAMP)-induced basal defenses (Kim et al. 2005). An alternative strategy employed by AvrPtoB from *P. syringae* pv. *tomato* and AvrPphC from *P. syringae* pv. *phaseolicola* is to suppress gene-for-gene induced hypersensitive response (HR) (Abramovitch et al. 2003; Tsiamis et al. 2000). Another important finding has been that most R proteins do not interact directly with pathogen effectors, but rather monitor changes to the virulence target of the effector. This has become known as the guard model (Dangle and Jones 2001). For example, RPS5 from *Arabidopsis* initiates the HR only after the serine/threonine kinase PBS1 has been cleaved by the AvrPphB effector (Shao et al. 2003).

The majority of *Avr* genes have been cloned from bacterial pathogens belonging to the genera *Pseudomonas* and *Xanthomonas*. However, a large number of important plant

diseases are caused by fungal and oomycete pathogens, which present a greater cloning challenge than bacteria due to their larger genomes (typically 10-40 Mb) and difficulty in culturing (as with the obligate biotrophs). Despite this, a number of *Avr* genes have been cloned from the oomycetes *Peronospora parasitica* (Allen et al. 2004; Rehmany et al. 2005) and *Phytophthora sojae* (Shan et al. 2004), as well as from the fungal pathogens *Melampsora lini* (Dodds et al. 2004), *Cladosporium fulvum* (Joosten et al. 1994; Luderer et al. 2002; Van Kan et al. 1991), *Magnaporthe grisea* (Orbach et al. 2000) and *Rynchosporium secalis* (Rohe et al. 1995).

Reverse genetics has been used successfully to clone the Avr2, Avr4 and Avr9 effectors from *C. fulvum* (Joosten et al. 1994; Luderer et al. 2002; Van Kan et al. 1991) and the Nip1 effector from *Rhynchosporium secalis* (Rohe et al. 1995). The remaining fungal and oomycete *Avr* genes were isolated using a map-based cloning approach. *Pyrenophora teres* is also amenable to map-based cloning since it has a heterothallic mating system, controlled by alternate alleles of the *MAT* gene (Rau et al. 2005), that is helpful for creating the mapping population necessary for map-based cloning. This study reports on the identification of amplified fragment length polymorphism (AFLP) markers linked to an avirulence gene in the *P. teres* isolate WRS 1906 that limits the ability of this isolate to infect the barley cultivar 'Heartland.' This and other work indicating that the *P. teres*-barley pathosystem conforms to the gene-for-gene model is discussed. Markers identified in this study will provide a staring point for map-based cloning of this avirulence gene.

Materials and Methods

Barley Lines and *Pyrenophora teres* Isolates

To establish the appropriate study system, it was necessary to identify two *P. teres* isolates that exhibited a differential ability to infect an individual host line. Seven cultivated barley (*Hordeum vulgare* ssp. *vulgare*) lines and one wild barley (*Hordeum*

vulgare ssp. *spontaneum*) accession were selected as differentials. A ninth line (Harbin), used in a previous evaluation of avirulence in *P. teres* (Weiland et al. 1999), was screened with the two isolates selected to form the mating population in this study, to determine if the avirulence locus in this study was the same as that previously reported. The lines used are listed in Table 2.1. Eight net form (*P. teres* f. *teres*) and two spot form (*P. teres* f. *maculata*) isolates were chosen to evaluate virulence on the differentials (Table 2.2).

Table 2.1. Barley lines used to evaluate virulence of net blotch isolates.

Species	Line	Origin
H. vulgare ssp. *vulgare*	Excel	University of Minnesota
	CDC Dolly	University of Saskatchewan
	Harbin	Manchurian landrace
	Harrington	University of Saskatchewan
	Heartland	Brandon Research Centre, AAFC[1]
	Norbert	Cereal Research Centre, AAFC
	TR251	Brandon Research Centre, AAFC
	TR473	University of Saskatchewan
H. vulgare ssp. *spontaneum*	Caesarea 26-24[2]	Israeli landrace

[1] Agriculture and Agri-Food Canada
[2] provided by P. Hayes (Oregon State University), collected by E. Nevo (University of Haifa)

Table 2.2. Net blotch isolates used in this study.

Form	Isolate	Collection Location	Collection Year
Net	SK 1	Scott, SK	2001
	SK 2	Lashburn, SK	2001
	SK 3	North Battleford, SK	2001
	WRS 102[1]	Indian Head, SK	1959
	WRS 858	Teulon, MB	1973
	WRS 1607	Prince Albert, SK	1985
	WRS 1906	Fredricton, NB	1994
	WRS 1907	PEI	1996
Spot	SK 4	North Battleford, SK	2001
	WRS 857	Oakbank, AB	1973

[1] all WRS isolates were provided by A. Tekauz (Cereal Research Centre, AAFC)

Virulence Phenotyping

Pyrenophora teres cultures used for virulence evaluation were initially established from single spores collected from infected barley leaves. Dry leaves infected with *P. teres* were surface sterilized in 50% ethanol for 15 s, 2% sodium hypochlorite for 30 s and then rinsed in distilled water. Leaf sections were placed on dry filter paper in Petri plates and a second, moistened piece of filter paper was attached to the lid. Plates were

incubated at 21ºC with a 12 h photoperiod under cool white light (15 W, 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photon flux, GE, Mississauga, ON) to promote sporulation. After 3-5 d, single conidia were transferred to Petri plates (9 cm diameter) containing V8A (18% V8 Juice (v/v), 0.3% $CaCO_3$, 2% Agar) using a sterile pipet tip (pulled to a fine point). Cultures were incubated for 10 d as above. Inoculum was prepared by washing conidia from plates with 10 mL of water and filtering through two layers of cheesecloth. Inoculum concentration was adjusted to 10^4 conidia/mL. A drop of Tween 20 was added to the suspension as a wetting agent. Subsequent cultures for inoculation were initiated by placing several crystals of silica gel-containing conidia onto V8A plates. Silica gel stocks were prepared from the initial single-spore derived cultures using the method of Smith and Onions (1994).

Barley seeds were surface sterilized in 0.12% formaldehyde for 1 h and then rinsed in running tap water for 30 min. Five seeds per line were planted in a group, with two groups per 15 cm pot. Pots contained Terra-lite Redi-Earth (W.R. Grace Ltd., Ajax, ON). Plants were grown at 21ºC with a 16 h photoperiod (75% RH) for 10-14 d and staked to prevent touching of leaves between pots. Inoculum was then applied to three-leaf stage plants with a 50 mL spray bottle. Inoculum was applied to run-off, approximately 7.5 mL per 15 cm pot. Plants were then incubated in darkness for 24 h at 100% RH. They were then returned to the previous conditions for one week before disease symptoms on the second leaf were rated on a 1-10 scale (Tekauz 1985). An interpretation of the net form pictographs from Tekauz (1985) for each numerical rating are as follows: 1, pinpoint necrotic lesions without a visible centre; 2, a mixture of pinpoint lesions and short linear necrotic lesions running parallel with the long axis of the leaf blade; 3, a mixture of pinpoint lesions and short linear lesions with some chlorosis evident around necrotic areas; 4, all short linear lesions with some chlorosis around necrotic areas; 5, short linear lesions with some short perpendicular linear necrosis and expanding chlorosis around necrotic tissue; 6, all necrotic lesions show "net" pattern with surrounding chlorosis; 7, more extensive net pattern of necrotic tissue and chlorosis, but lesions are still isolated from one another; 8, lesions begin to coalesce; 9, extensive necrotic tissue and entire leaf is chlorotic; 10, greater levels of necrosis and

some areas demonstrate a blue/gray color. The spot form symptoms are similar except the necrotic areas are round to oval instead of netted. Lesions rated 5 or lower remain restricted in size and are considered resistant reactions, while lesions rated above 5 continue to expand over time and are considered susceptible reactions. Therefore, isolates that produced a rating of ≥6 were classified as virulent while those eliciting a reaction of ≤5 were considered avirulent. Four independent inoculations on each differential line were carried out on different inoculation dates with the eight isolates initially evaluated. Subsequently, three independent inoculations on different inoculation dates were carried out with each isolate in the mapping population selected for *Avr* gene identification on the corresponding differential line.

Pyrenophora teres Mating and Mating-Type Determination

Barley stem sections, including the leaf sheath, were cut into 3-4 cm pieces and autoclaved at 18 psi for 30 min. Stem pieces were transferred to Petri plates containing 35-40 mL of Sach's Nutrient Agar (0.1% $Ca(NO_3)_2 \cdot 4H_2O$, 0.025% KCl, 0.025% K_2HPO_4, 0.025% $MgSO_4 \cdot 7H_2O$, 0.001% $FeC_6H_5O_7 \cdot 5H_2O$, 0.4% $CaCO_3$, 2% Agar, pH to 5.95 with H_3PO_4) with 1 cm separating each piece (5-6 pieces/plate). Isolates were cultured separately on V8A at 21ºC with a 12 h photoperiod until abundant conidia were produced (~10 d). Conidia were collected from the plates as previously described and the suspension was adjusted to 10^4 conidia/mL. A 25 µL aliquot of each isolate was pipeted onto opposite ends of each straw piece to initiate mating. Plates were sealed with parafilm and placed in the dark at 15ºC. After 12 weeks the plates were transferred to a 9 h photoperiod with an increase of 3 min/d for 3 weeks. Mature pseudothecia were produced at this point. Mature asci were released from the pseudothecia by crushing with a sterile needle, acospores were released from the asci by crushing with a finely drawn glass pipet tip. Individual ascospores (primarily random meiotic products along with complete tetrads when possible) were transferred to 5% water agar plates to germinate overnight at 25ºC. Germinated ascospores were transferred to V8A plates to produce conidia that were stored as silica gel stocks.

Mating-type was determined using PCR primers specific to the *MAT-1* and *MAT-2* alleles (Rau et al. 2005). The *MAT-1* primers were 5'-AAC AGA CTC CTC TTG ACA ACC CG-3' (forward) and 5'-TGA CGA TGC ATA GTT TGT AAG GGT-3' (reverse), and the *MAT-2* primers were 5'-CAA CTT TTC TCT ACC ACA CGT ATC CC-3' (forward) and 5'-TGT GGC GAT GCA TAG TTC GTA C-3' (reverse). PCR reactions were carried out in 20 µL reactions and contained 1× buffer (20 mM Tris HCl (pH 8.4), 50 mM KCl), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 4 pmol of each primer, 1 U *Taq* polymerase and 20 ng of DNA. PCR conditions consisted of 35 cycles of 94ºC for 30 s, 56ºC for 30 s and 72ºC for 30 s, followed by a final hold at 72ºC for 1 min. PCR products were loaded on 1% agarose gels containing 0.1 µg/mL ethidium bromide and electrophoresed in 0.5× TBE for 2 h at 115 V. Gels were photographed under UV light.

DNA Extraction and Bulked Segregant Analysis

Conidia were harvested from 10-14 d cultures grown on V8A and added to 75 mL Liquid Medium (LM) (0.25% $MgSO_4 \cdot 7H_2O$, 0.27% KH_2PO_4, 0.1% Bacto-Peptone, 0.1% Yeast Extract, 1.0% Sucrose) in a 250 mL flask. Flasks were shaken at 150 rpm at 25ºC with constant light for 24 h. Mycelium was harvested by centrifugation at 2800× *g* for 8 min at 25°C, rinsed once with ddH_2O, spun again and placed at -80°C overnight. Samples were freeze-dried and ground for 3 min with glass beads using an automated shaker. To each sample, 20 mL of warm (65°C) Extraction Buffer (EB) (0.5 M NaCl, 100 mM Tris-Cl (pH 8.0), 50 mM EDTA (pH 8.0), 1.25% SDS, plus 3.8 g sodium bisulfite just before use) was added, mixed and incubated at 55-60°C for 30 min. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the tubes, mixed vigorously and centrifuged for 15 min at 250× *g* at 25°C. The upper phase was transferred to a new 50 mL tube. Two volumes of (-20°C) 95% ethanol was added, mixed gently to precipitate the DNA and placed at -20°C for 30-60 min. DNA was hooked on a Pasteur pipet, transferred to a new 50 mL tube and washed with 30 mL (-20°C) 70% ethanol. After removing the 70% ethanol, the pellet was dissolved in 2 mL TE (pH 8.0) by incubation at 60°C. After adding 1 mL of 7.5 M ammonium acetate, the

tubes were spun for 20 min at 16 000 × *g*. The supernatant was transferred to a new tube and 20 mL of (-20°C) 95% ethanol was added, left for 30 min at -20°C and the DNA hooked into a microfuge tube. The tube was centrifuged for 10 min at 16 000× *g*, the 95% ethanol removed, washed in 70% ethanol and left to dry upside down for 30 min in a fumehood. The pellet was dissolved in 200-400 µL of sterile H_2O. To quantify DNA concentrations, a 1:50 dilution of DNA was made and compared to a series of lambda DNA dilutions (5, 10, 20, 30, 40, 50 ng/µL). Samples were run on 1.2% 0.5× TBE agarose gels for 2 h at 115V.

Bulked segregant analysis (BSA) (Michelmore et al. 1991) was carried out with 30 progeny isolates derived from the cross WRS 1906 × WRS 1607. Equal concentrations of DNA from 15 virulent isolates were bulked into three pools containing five isolates each. Three similar pools were also constructed using DNA from 15 avirulent isolates. These six pools were then used to identify AFLP markers linked to the avirulence gene. Any markers found linked to the pools were then screened against the entire mapping population.

AFLP Analysis

AFLP was carried out using a method modified from Vos et al. (1995). 250 ng of DNA was used as the starting template. Primers containing only one selective base (E-A, E-C, E-G, M-A, M-C, M-T) were used for the pre-selective amplification. Twelve *Eco*RI primers containing two selective bases (E-AA, E-AC, E-AG, E-AT, E-CA, E-CC, E-CG, E-CT, E-GEA, E-GC, E-GG, E-GT) and twelve *Mse*I primers containing two selective bases (M-AA, M-AC, M-AG, M-AT, M-CA, M-CC, M-CG, M-CT, M-TA, M-TC, M-TG, M-TT) were used in all combinations for selective amplification reactions. Primer pairs showing a large number of polymorphisms between parental isolates were used to screen the bulked DNA pools. AFLP bands were run on 6% polyacrylamide gels and visualized by silver staining. Markers were named using the four extension letters from the selective primers and the band size. For example, the marker AACT305 denotes a band of 305 bp produced with the primer pair E-AA/M-CT.

Data Analysis and Linkage Group Construction

All virulence phenotypic data and AFLP markers identified by BSA were tested for deviations from the expected 1:1 segregation ratio using the χ^2 test ($\alpha = 0.05$). For the purposes of mapping, virulence ratings similar to the low virulence parent and markers originating from this parent were scored as 'a' while virulence similar to the high virulent parent and markers originating from this parent were scored as 'b.' JoinMap 3.0 (Van Ooijen and Voorrips 2001) was used to group and order the markers and virulence phenotype data. The linkage groups were evaluated at several LOD scores (ranging from 2.0 to 5.0). A LOD of 4.0 was determined to be a suitable significance level because the group did not change at higher LOD scores. The linkage group was then ordered (chi-square jump restriction = 5, maximum recombination value = 0.5, minimum LOD = 1.0) and a goodness-of-fit (ripple) performed after the addition of each new marker to the growing linkage group. Linkage between ordered loci was calculated using the Kosambi mapping function (Kosambi 1944).

Results

Virulence Variability of *Pyrenophora teres* Isolates

All isolates used in this study produced abundant conidia in culture (Figure 2.1A and 2.1B). Virulence of the *P. teres* isolates on the barley differential lines is summarized in Table 2.3. Reactions ranged from highly virulent to highly avirulent, with no one isolate being virulent, or avirulent, on all lines. Typical net form and spot form reactions are shown in Figure 2.2. Incompatible reactions were observed most often with Heartland (7 of 10 isolates), while compatible reactions were recorded on Harrington with all isolates tested. Disease ratings across replications did not differ by more than two units.

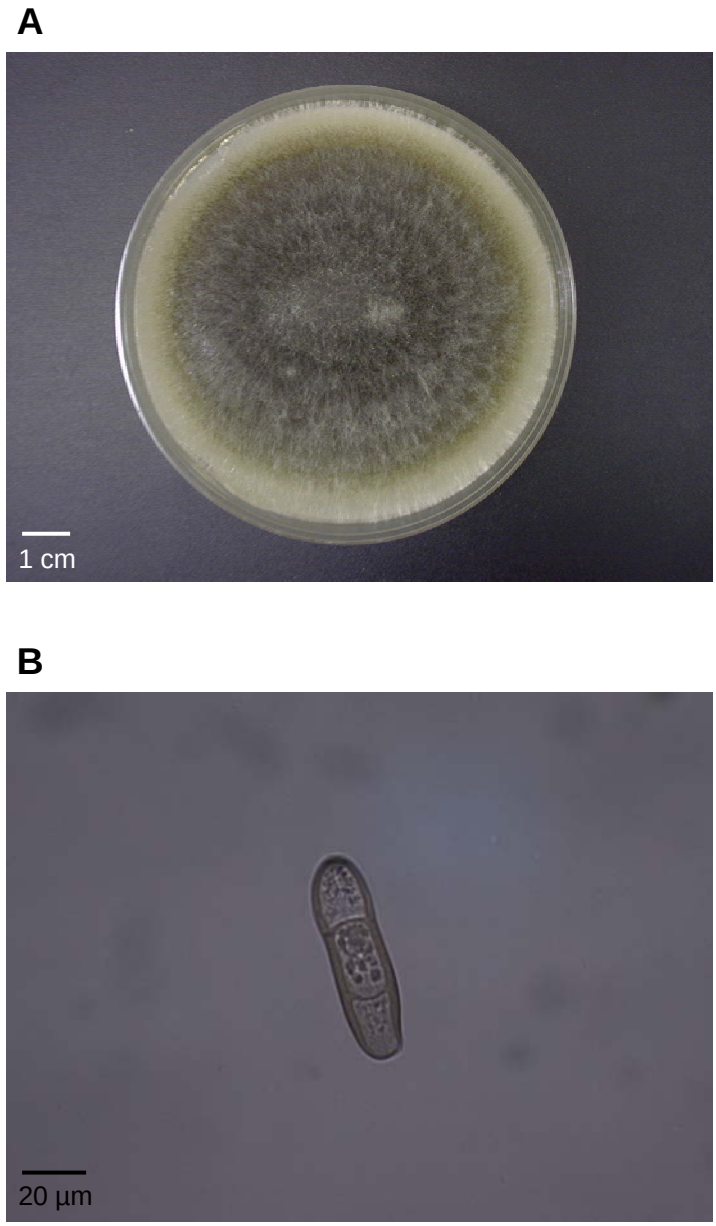

Figure 2.1. Morphological characteristics of *Pyrenophora teres*. (A) Culture grown on V8A, pigmented areas are the conidia. No differences were observed between net or spot form isolates. (B) Isolated conidium of *Pyrenophora teres*, again no morphological variation was observed between net or spot form isolates.

Figure 2.2. Typical spot (left) and net (right) symptoms elicited on barley leaves by the two forms of *Pyrenophora teres*.

Table 2.3. Virulence phenotypes of ten *Pyrenophora teres* isolates on nine differential barley lines.

	Barley Line								
Isolate	Excel	CDC Dolly	Harbin	Harrington	Heartland	Norbert	TR251	TR473	Caesarea 26-24
SK 1	2 (3)[1]	7	-	9	1 (2)	7 (8)	4 (5)	7 (6,8)	7 (6,8)
SK 2	2 (1,3)	8 (7)	-	9	1	8	7 (6,8)	7 (8)	4
SK 3	2 (1,3)	3 (4)	-	9	3 (5)	8	8 (6)	4 (6)	4
WRS 102	2 (3)	2	-	8 (7,9)	2 (3)	2 (4)	2	2 (3)	7
WRS 858	2 (1,3)	3 (4)	-	9	7 (6,8)	7 (6,8)	2 (1,3)	3 (4)	3
WRS 1607	6 (8)	5	8 (7)	9 (8)	8 (9)	5 (4,6)	1 (2)	3 (5)	8 (7,9)
WRS 1906	6 (5)	5	7 (8)	8 (9)	1 (2)	6 (5,7)	2 (3)	5 (4)	3
WRS 1907	5 (4)	1 (2)	-	8 (7,9)	2 (1)	1 (3)	2	2 (1)	3 (2,4)
SK 4	9 (8)	9	-	8 (7,9)	7	9	2 (1)	9	5 (4)
WRS 857	7 (6)	8 (7)	-	9 (8)	2 (1)	7	2 (1)	7 (6,8)	2 (1)

[1] The most frequent rating (mode) is reported with other observed ratings in parentheses.

Pyrenophora teres Mating and Phenotyping of Mapping Population

Matings between all possible biparental combinations of the same form were established (Figure 2.3A). To confirm the self-incompatibility of this pathogen, crosses using a single isolate were also attempted. The production of pseudothecia and ascospores was observed in a number of net by net crosses (Figures 2.3B and 2.3C), but no successful crosses involving a spot form isolate were observed (Table 2.4). None of the matings involving a single isolate produced any ascospores.

Table 2.4. Mating-type determination and crossing results for *Pyrenophora teres* isolates used in this study.

Isolate	Mating-Type	Successful Crosses	Self Compatible
SK 1	MAT-2	SK 2, WRS 858, WRS 1607	-
SK 2	MAT-1	SK 1, SK 3, WRS 102, WRS 1906, WRS 1907	-
SK 3	MAT-2	SK 2, WRS 858, WRS 1607	-
WRS 102	MAT-2	SK 2, WRS 858, WRS 1607	-
WRS 858	MAT-1	SK 1, SK 3, WRS 102, WRS 1906, WRS 1907	-
WRS 1607	MAT-1	SK 1, SK 3, WRS 102, WRS 1906, WRS 1907	-
WRS 1906	MAT-2	SK 2, WRS 858, WRS 1607	-
WRS 1907	MAT-2	SK 2, WRS 858, WRS 1607	-
SK 4	MAT-2	None	-
WRS 857	MAT-2	None	-

The *MAT-1*/*MAT-2* PCR assay confirmed that successful crosses occurred between isolates with alternate mating-type alleles, while unsuccessful crosses resulted from isolates having the same mating-type allele (Table 2.4).

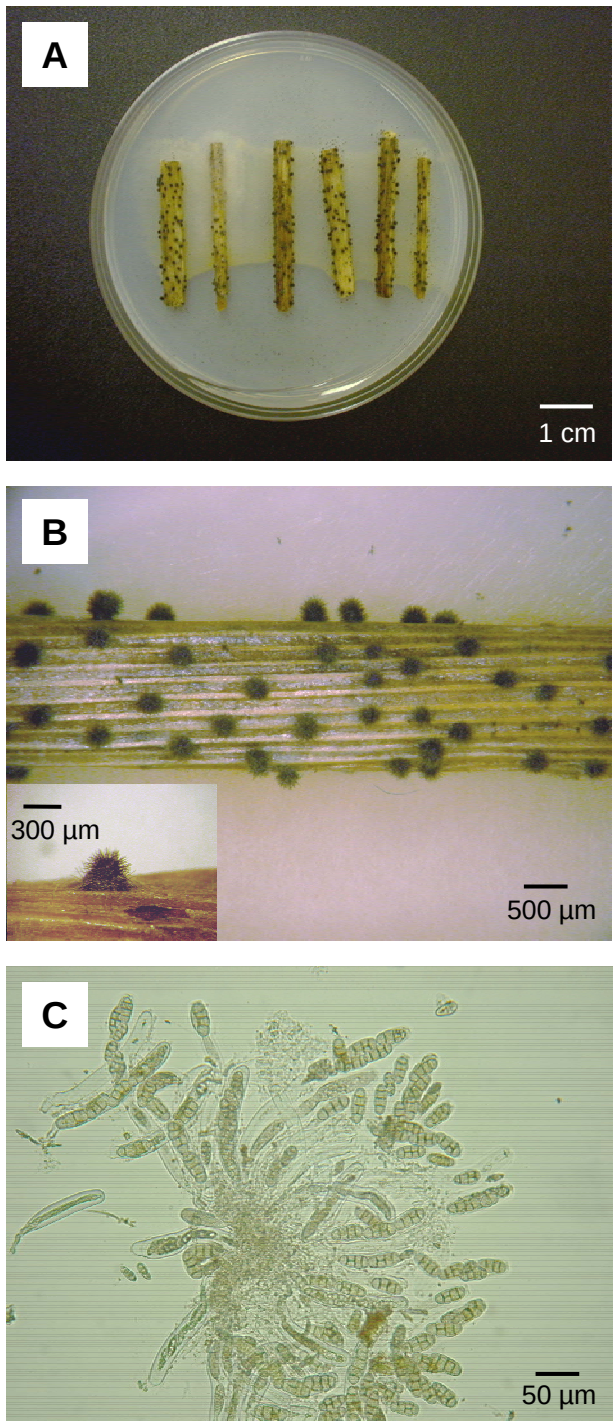

Figure 2.3. Culture system used to mate *Pyrenophora teres* isolates. (A) Sterilized barley stems on SNA plates. (B) Formation of pseudothecia on a barley stem and magnified view of individual pseudothecia (inset). (C) Production of ascospores from successful mating.

The cross between WRS 1906 and WRS 1607 was selected to generate a mapping population to identify an avirulence gene. These parental isolates were selected because of their mating compatibility, the large number of ascospores produced from the cross, and their contrasting virulence on the differential line Heartland (Figure 2.4A). Additionally, both isolates were virulent on Harbin (Table 2.3) indicating that the locus controlling avirulence in WRS 1906 against Heartland is not the same as the avirulence locus identified in a previous study by Weiland et al. (1999) (explained later). Because both parents were highly virulent on Harrington (Figure 2.4B), this barley line could be used as a positive control when phenotyping the mapping population. A total of 93 single ascospore progeny were originally isolated from this cross, but this number was reduced to 67 after some ascospores failed to germinate and others were identified as clones. The population segregated 34 avirulent: 33 virulent ($\chi^2 = 0.0$, $P = 1.0$) (Figure 2.5) when phenotyped for virulence on Heartland suggesting single gene control of the avirulent phenotype. This 1:1 ratio was also confirmed when a complete set of ascospores from the same ascus segregated four avirulent to four virulent. All isolates were highly virulent on Harrington (data not shown).

AFLP Markers Linked to the Avirulence Gene

Parental isolates WRS 1906 and WRS 1607 were screened with 144 AFLP primer pairs producing an average of 40 bands per reaction and 3.6 polymorphisms between the parents. Twenty-three primer pairs were selected based on the high number of scorable polymorphic bands produced and the diversity of selective bases in the primers. From these 23 primer pairs six bands were identified by BSA as linked to the avirulence phenotype (Figure 2.6). Five were derived from WRS 1607 and one from WRS 1906 (Table 2.5). When these bands (markers) were screened across the entire mapping population all segregated in a 1:1 Mendelian ratio (Table 2.5). The linkage group produced extends 20 cM with five of the markers located to one side of the avirulence locus and a single marker on the other (Figure 2.7). The avirulence locus was designated $Avr_{Heartland}$.

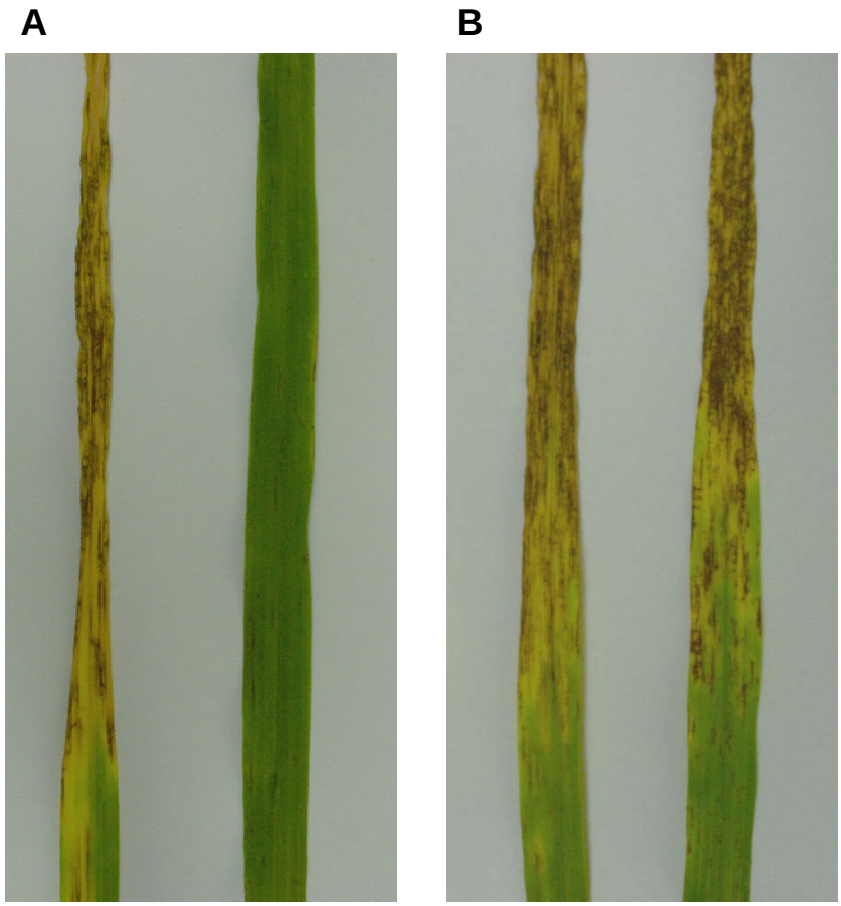

Figure 2.4. Virulence of *Pyrenophora teres* parental isolates, WRS 1906 and WRS 1607, used to create the mapping population. (A) Differential reactions on Heartland. (B) Reactions on the control Harrington.

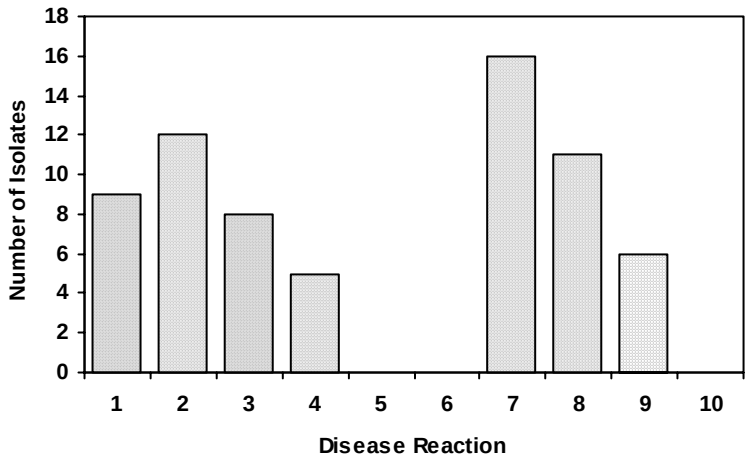

Figure 2.5. Disease reactions incited on the barley differential line Heartland by *Pyrenophora teres* isolates of the mapping population derived from the WRS 1906 × WRS 1607 cross. Isolates that produced a rating of ≥ 6 were classified as virulent while isolates eliciting a reaction of ≤ 5 were considered avirulent. WRS 1906 elicited a rating of 1 while WRS 1607 elicited a rating of 8.

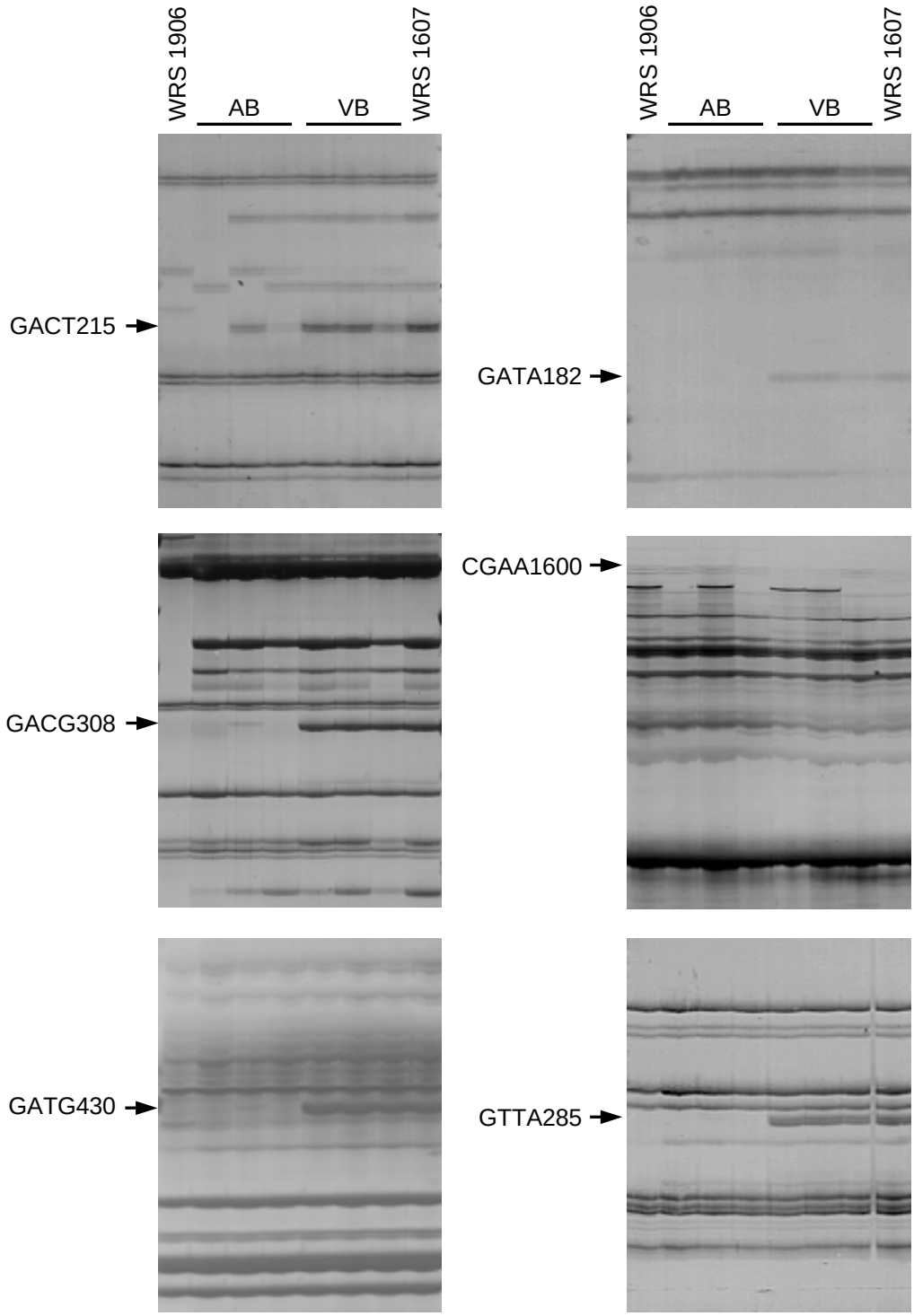

Figure 2.6. Identification of AFLP markers linked to the *Pyrenophora teres* avirulence gene ($Avr_{Heartland}$) by BSA. AB are the avirulent bulks, VB are the virulent bulks, and the name of the marker is listed to the left of each image.

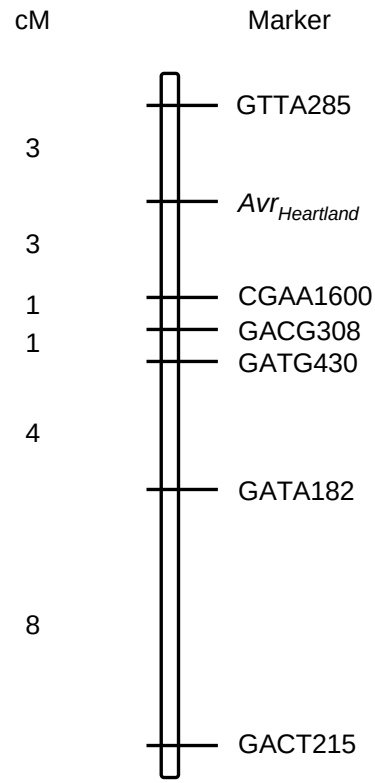

Figure 2.7. Linkage group encompassing the *Pyrenophora teres* avirulence gene ($Avr_{Heartland}$) controlling incompatibility on the barley line Heartland. Recombination units are indicated on the left and marker names are shown on the right.

Table 2.5. Data for AFLP markers linked to the *Pyrenophora teres* avirulence locus ($Avr_{Heartland}$).

Marker	Segregation Ratio[1]	χ^2	P	Parental Origin
GACT215	35:32	0.059	0.8069	WRS 1607
GATA182	34:33	0	1	WRS 1607
GATG430	34:33	0	1	WRS 1607
GACG308	35:32	0.059	0.8069	WRS 1607
CGAA1600	36:31	0.239	0.6250	WRS 1906
GTTA285	32:35	0.239	0.6250	WRS 1607

[1] WRS 1906 genotype:WRS 1607 genotype

Discussion

The 1:1 segregation ratio for virulence on Heartland observed for the mapping population derived from WRS 1906 × WRS 1607 provides evidence that a single gene (designated $Avr_{Heartland}$) in WRS 1906 is responsible for determining low virulence on this barley line. This observation is similar to a previous report by Weiland et al. (1999) in which a single gene was also identified in a cross between isolates 0-1 and 15A controlling avirulence of 15A on the barley line Harbin. However, the gene identified in this study is not the same as that located by Weiland et al. (1999) because both WRS 1906 and WRS 1607 are virulent on Harbin.

Identification of a second *Avr* gene in *P. teres* provides strong support that this pathogen's interaction with barley is controlled in a gene-for-gene manner. This is in contrast to the *P. tritici-repentis*-wheat pathosystem which follows a toxin model of interaction (Strelkov and Lamari 2003). Interestingly, *P. tritici-repentis* is the only pathogen which produces multiple host-selective toxins (HSTs) against a single host species (Lamari et al. 2003). Eight races of *P. tritici-repentis* have been described based on their virulence against three differentials (Strelkov and Lamari 2003). This virulence is controlled by the presence or absence of three toxins that produce either chlorotic or necrotic symptoms.

Initially, three toxins were isolated from *P. teres* cultures (Bach et al. 1979). However, subsequent reports have shown that *P. teres* produces only one toxin (ToxC) (Friis et al. 1991). ToxA was demonstrated to be a precursor of ToxC, and ToxB was an artefact generated from ToxC when culture conditions changed (Friis et al. 1991). Therefore, the presence of two distinct genes controlling host line-specific interactions rules out the possibility that both produce toxins that could conceivably form the basis of a race system similar to *P. tritici-repentis*. Additionally, Reiss and Bryngelsson (1996) noted that, while toxin application to detached leaves mimicked net blotch symptoms, cultivar-specific resistance was lost, indicating that the toxins alone did not account for interaction specificity with barley. It therefore appears that this toxin is a virulence factor, in contrast to HSTs which are pathogenicity factors, further support for the gene-for-gene model in this pathosystem.

The existence of multiple *Avr* genes has been reported in numerous pathogenic fungi and oomycetes. Examples include, three genetically independent *Avr* genes mapped in *Magnaporthe grisea* (Dioh et al. 2000), seven in *Blumeria graminis* (Pedersen et al. 2002), and six in *Phytophthora infestans* (Van der Lee et al. 2001). Cloning of *Avr* genes from fungi and oomycetes has revealed allelic polymorphism and clustering of *Avr* genes in addition to independent genetic loci. This variability results in specific recognition by different *R* genes. For example, Dodds et al. (2004) cloned three clustered *Avr* genes in *M. lini*, *AvrL567-A, -B* and *–C*, from an 11.5 kb region and demonstrated that the *AvrL567-A* and *-B* genes are specifically recognized by the *L5*, *L6* and *L7* flax resistance genes, but not by other *L* locus genes. Rehmany et al. (2005) identified six alleles of the *P. parasitica* ATR^{NdWsB} gene which were differentially recognized by *RPP1* genes present in two accessions of *Arabidopsis*. Although barley resistance genes against net blotch have not been analyzed to determine levels of allelism and clustering that may be present, the presence of several major *R* genes (Graner et al. 1996; Manninen et al. 2000; Williams et al. 1999) indicates that specific *R* gene/alleles may recognize distinct *P. teres Avr* genes/alleles.

No two *P. teres* isolates evaluated in this study showed the same compatibility/incompatibility pattern across the barley differentials. This may be a reflection of the selective pressure imposed on *P. teres* populations by different barley cultivars (and their *R* genes) grown across the diverse range of locations and years from which the isolates were collected. Unfortunately this is difficult to assess since the original host cultivars are unknown. However, diverse pathotype populations have been reported in other studies. For example, 45 net form and 20 spot form pathotypes were identified on nine and 12 differential lines, respectively, in western Canada (Tekauz 1990), 80 net form pathotypes were reported in the USSR using seven differentials (Afanasenko and Levitin 1979) and 13 pathotypes were identified in California using 22 differential lines (Steffenson and Webster 1992).

The diversity of *P. teres* pathotypes reported is likely the result of sexual reproduction. A recent study by Rau et al. (2005) concluded that sexual reproduction was occurring in field populations of *P. teres*. They found that both mating-types existed in a 1:1 Mendelian ratio in all six populations sampled. Such ratios, along with genotypic diversity (Peever and Milgroom 1994) and gametic equilibrium (Rau et al. 2003) reported in *P. teres*, are typical indicators of randomly mating populations (Milgroom 1996). The implications of sexual reproduction as it applies to virulence and pathotype generation has not been investigated specifically in *P. teres*, however, pathogens which incorporate sexual reproduction as part of their lifecycle (especially obligate outcrossing), combined with production of abundant asexual spores and high potential gene flow, represent a high risk to *R* genes (McDonald and Linde 2002). *P. teres* meets these criteria.

Sexual reproduction impacts pathotype generation by not only disseminating mutations that affect virulence, but the reshuffling of genes during meiosis increases the probability of creating new virulence pathotypes (McDonald and Linde 2002). Several studies have shown that virulent strains of a pathogen harbour mutated forms of the normal *Avr* gene. For example, analysis of *M. grisea* strains virulent against the rice *Pi-ta* gene showed a variety of point mutations, resulting in premature stop codons, and

deletion/insertion events (including complete loss of the gene) associated with the *AVR-Pita* gene (Orbach et al. 2000).

This study identified AFLP markers linked to a *P. teres* avirulence gene ($Avr_{Heartland}$) which will provide a starting point for map-based cloning of this gene. Characterization of this gene and its encoded protein would establish a role in virulence and provide a valuable comparison to the *P. tritici-repentis* toxins. In no other genera do distinct pathogenic species follow both the toxin and gene-for-gene models. This gene is being mapped onto a genetic map of *P. teres* so that future mapping of *Avr* genes can clearly establish their relationship to one another and more firmly determine the relationship between specific barley *R* genes and *P. teres Avr* genes.

Chapter 3: Karyotype Analysis of the Net and Spot Forms of *Pyrenophora teres*

Abstract

Five isolates of the net blotch pathogen, *Pyrenophora teres*, representing both the net and spot forms were analyzed by the germ tube burst method (GTBM) and pulsed field gel electrophoresis (PFGE) to determine the species' karyotype. Nine chromosomes were observed from all isolates using the GTBM and estimation of chromosome lengths varied from 0.5 to 3.0 μm. PFGE separated 6 or 8 bands depending on the isolate, but analysis of band intensity and size indicated nine chromosomes. Chromosome sizes ranged from 1.8 to ~6.0 Mb providing a genome size estimate of 34 to 37 Mb. Significant chromosome-length polymorphisms (CLP) were observed among isolates. These CLP did not hinder mating between mating-type compatible net form isolates. No particular CLP or individual chromosome could be associated with differences in disease symptoms observed between pathogen forms. This study provides the first karyotypes of both *P. teres* forms and will assist genetic mapping of this pathogen.

Introduction

Karyotype analysis provides fundamental information about the genome organization of a species. Traditional karyotyping in higher eukaryotes relies on microscopic examination of mitotic stage chromosomes (Taga et al. 1998). This approach has not been widely applied to fungi because not only are their chromosomes extremely small, but observations of mitotic chromosomes within mycelia are hindered by the presence of the cell wall, the nuclear membrane which remains partially intact throughout division, and the confined space which prevents adequate chromosome spreading (Lu 1996).

Successful fungal karyotyping based on cytological observations was initiated in *Neurospora crassa* by McClintock (1945) focussing on meiotic chromosomes because

the large ascospores allowed better chromosome separation. However, the possibility of observing meiotic chromosomes is not available for many fungal species for which the sexual stage is unknown or difficult to induce.

In response to difficulties associated with cytological karyotyping, Shirane et al. (1988) developed the germ tube burst method (GTBM). The GTBM is a simple procedure which releases mitotic metaphase chromosomes from the actively growing end of young conidial germ tubes by exposing the fungus to an alcohol-based hypotonic solution. This method is not limited to fungi with a sexual stage and observations of chromosomes are much clearer due to the absence of interfering cellular structures. Karyotype analysis using the GTBM has been performed with a limited number of fungi including five species of *Botrytis* (Shirane et al. 1989), *Fusarium graminearum* (Gale et al. 2005), *Nectria haematococca* (Taga et al. 1999), *Alternaria alternata* (Akamatsu et al. 1999) and three *Cochliobolus* species (Tsuchiya and Taga 2001).

Pulsed field gel electrophoresis (PFGE) has been a more popular technique for karyotyping fungi. To date, over 100 species have been analysed using this method (Beadle et al. 2003). PFGE allows separation of DNA molecules under 10 Mb, which is ideal for the small fungal chromosomes. It can also be applied to all species of fungi and provides a rough estimate of genome size. However, accurate estimates of chromosome number and genome size are difficult if chromosomes are of similar size, as is often the case, and therefore not resolvable in the gel. The combined use of the GTBM and PFGE in species such as *N. haematococca* (Taga et al. 1998) and *F. graminearum* (Gale et al. 2005), has proven a reliable strategy for obtaining accurate karyotypes.

Pyrenophora teres Drechs. (anamorph: *Drechslera teres* (Sacc.) Shoem.) is the causative agent of barley net blotch. It is an economically important pathogen commonly found in all major barley producing regions of the world and can cause yield losses ranging from 15-35% (Khan 1987; Martin 1985; Steffenson et al. 1991). The pathogen exists as two distinct forms which are morphologically identical, but elicit pathologically distinct

symptoms on barley. The net form (*P. teres* f. *teres*) produces elongated light-brown lesions with dark-brown reticulation within the lesion and surrounded by chlorotic tissue, while the spot form (*P. teres* f. *maculata*) produces dark brown elliptical lesions surrounded by chlorotic tissue. Little is known about the genetic organization of the two forms and how they compare to one another. This paper reports on the karyotype analysis of isolates representing both the net and spot forms of the pathogen using the GTBM and PFGE, and discusses these findings with respect to differences between the two pathogen forms. This is the first reported use of the GTBM in this species and, in combination with PFGE, provides the first karyotype of both forms.

Materials and Methods

Pyrenophora teres Isolates

Net blotch isolates used in this study are listed in Table 3.1. They were cultured on V8A (18% V8 Juice (v/v), 0.3% $CaCO_3$, 2% Agar) plates at 21ºC with a 12 h photoperiod under cool white light (15 Watts, 760 lumens, General Electric, Mississauga, ON) to induce sporulation.

Table 3.1. *Pyrenophora teres* isolates used in this study.

Form	Isolate	Collection Location	Collection Year	Mating-Type
Net	SK 2	Lashburn, SK	2001	MAT-1
	WRS 1607[1]	Prince Albert, SK	1985	MAT-1
	WRS 1906	Fredricton, NB	1994	MAT-2
Spot	SK 4	North Battleford, SK	2001	MAT-2
	WRS 857	Oakbank, AB	1973	MAT-2

[1] all WRS isolates were provided by A. Tekauz (Cereal Research Centre, AAFC)

Analysis of Germination Rate and Metaphase

Conidia were washed with 10 mL of water from 10 d cultures grown on V8A plates, filtered through two layers of cheesecloth and centrifuged at 2800× *g* for 7 min. Conidia were washed twice in potato dextrose broth (PDB) and resuspended in PDB at a concentration of 1×10^5/mL. Aliquots of 100 µL were pipetted onto poly-L-lysine-

coated slides (Sigma-Aldrich Corp., St. Louis, MO) and then placed into Petri dishes with two moist pieces of Whatman no. 3 filter paper applied to the base and lid. Plates were incubated at 25°C and removed at 10 min intervals over the course of 320 min. Slides were dried for 5 min, fixed in 3% formaldehyde/100mM $NaPO_4$ (pH 7.0) for 3 min, rinsed in phosphate buffer and left to dry for 30 min. Slides were stained for 5 min in the dark with a 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) (1 µg/mL)/Flourescent Brightener 28 (Sigma-Aldrich) (1 µg/mL) solution, rinsed in water and then mounted in antifade mounting solution (Johnson and Nogueira Araujo 1981). Slides were examined using a Zeiss Axiophot epiflourescent microscope and images captured using a Micropublisher 3.3 RTV camera (QImaging, Burnaby, BC) and Northern Eclipse v 7.0 digital imager software (Empix Imaging Inc., Mississauga, ON). Germination rate was recorded until complete germination was reached, at which point observations on the proportion of germ tubes in metaphase were recorded. Over one hundred conidia (for germination rate) or germ tubes (for metaphase proportion) were analyzed at each time point.

Hydroxyurea Treatment

To determine if hydroxyurea (an inhibitor of DNA synthesis) could synchronize cell division and increase the proportion of cells in metaphase, a 1 mL conidia suspension was amended with 100 mM hydroxyurea (Sigma-Aldrich) and 100 µL amounts were placed onto slides and incubated for 240 min at 25°C as described above. Slides were removed, shaken at 135 rpm for 5 min in water to wash away the hydroxyurea and 200 µL of fresh PDB was added to each slide before resuming the incubation. Slides were removed at 10 min intervals (up to 320 min), fixed in formaldehyde, stained with DAPI and observed as described above.

Germ Tube Burst Method

Conidia were prepared and incubated in the same manner as the analysis of germination rate experiment. Samples were collected at 10 min intervals over the time period at

which maximum metaphase was observed (from approximately 190 to 220 min). Slides were dipped in distilled water to remove the PDB and then fixed in a methanol/acetic acid (22:3, v/v) solution for 20 min. After flame drying the slides, samples were stained with DAPI and evaluated as described above. A total of 30 chromosome spreads were analyzed for each isolate.

Protoplast Isolation and Pulsed Field Gel Electrophoresis

Conidia were harvested from 10 d cultures grown on V8A and added to 75 mL Liquid Medium (0.25% $MgSO_4 \cdot 7H_2O$, 0.27% KH_2PO_4, 0.1% Bacto-Peptone, 0.1% Yeast Extract, 1.0% Sucrose) in 250 mL flasks. Flasks were shaken at 185 rpm at 25°C with constant light for 24 h. Mycelium was harvested by centrifugation at 2800× *g* for 8 min at 25°C and then rinsed with 100 mL ddH_2O over cheesecloth. Approximately 1.5-2.0 g (wet weight) of mycelia was placed into a 15 mL tube containing 6 mL of filter sterilized (0.22 µm) Digestion Buffer (40 mg/mL Driselase (Sigma), 0.8 mg/mL Chitinase (Sigma), 7 mg/mL β-Glucuronidase (Sigma) in 0.7 M NaCl). Tubes were shaken at 90 rpm at 28°C for 3-4 h. Protoplasts were filtered successively through one layer of cheesecloth and a 30 µm nylon filter (Morgans Filters Ltd., Pickering, ON), centrifuged at 2800× *g* for 10 min at 4°C, washed once in 0.7 M NaCl and pelleted again. Protoplasts were resuspended in a small volume of SE (1 M Sorbitiol, 50 mM EDTA (pH 8.0)) and mixed with an equal volume of 1.5% low melting point agarose (Bio-Rad Inc., Hercules, CA) dissolved in SE. The final concentration of protoplasts was 1×10^8/mL. After plugs solidified they were placed in 20 mL NDS (0.5 M EDTA (pH 9.1), 1% Sarkosyl, 10 mM Tris (pH 9.5), 1 mg/mL Proteinase K (Invitrogen Corp., Burlington, ON)) at 50°C for 24 h. Plugs were incubated in 0.5 M EDTA (pH 8.0) once at 50°C for 1 h, once at 25°C for 1 h and then stored in 0.5 M EDTA (pH 8.0) at 4°C.

PFGE was performed on the CHEF DR-III system (Bio-Rad). Separation of chromosomes in the 1-6 Mb range was carried out in 0.5× TBE at 14°C using 0.8% agarose gels (pulsed field certified agarose, Bio-Rad) with the following conditions: 1.5 V/cm, 120 h, 3500-1700 s; 1.5 V/cm, 24 h, 1700-1300 s; 2.0 V/cm, 24 h, 1300-800 s;

2.5 V/cm, 24 h, 800-600 s. *Schizosaccharomyces pombe* and *Hansenula wingei* chromosomes (Bio-Rad) were used as molecular size markers. Gels were stained in 0.5 µg/mL ethidium bromide for 1 h, destained in distilled water for 1 h and photographed under UV light. Densitometry readings of PFGE bands were taken using Quantity One v. 4.6.1 1-D analysis software (Bio-Rad) to determine the probable number of chromosomes migrating within one band.

Results

Germination, Nuclei and Metaphase Observations

Conidia began to germinate approximately 30 min after the start of incubation and nearly 100% germination was reached within 2 h. The time course for isolate WRS 1607 is shown in Figure 3.1 and is representative of all isolates used in the study. Nuclei, cell walls and septa were discernible under fluorescent light (Figure 3.2A). Although the specific stages of mitosis could not be distinguished, interphase nuclei (Figure 3.2A) were easily differentiated from metaphase nuclei (Figure 3.2B). Nuclei appeared to divide synchronously within a cell.

The proportion of nuclei in metaphase never exceeded 10% (Figure 3.1). There was a clear cycle in the frequency of metaphase, with peaks occurring at 60-70 min intervals. Hydroxyurea treatment had no effect on metaphase frequency. Cells resumed division approximately 20 min after the hydroxyurea was removed from the incubation media, however, there was no increase in the percentage of metaphase nuclei (Figure 3.1).

Karyotype Analysis

The GTBM was used on conidia incubated for 280 min (the maximum metaphase frequency observed). The method successfully burst the germ tubes, releasing the nuclei from the terminal hyphal cell (Figure 3.3). Densely staining heterochromatin was

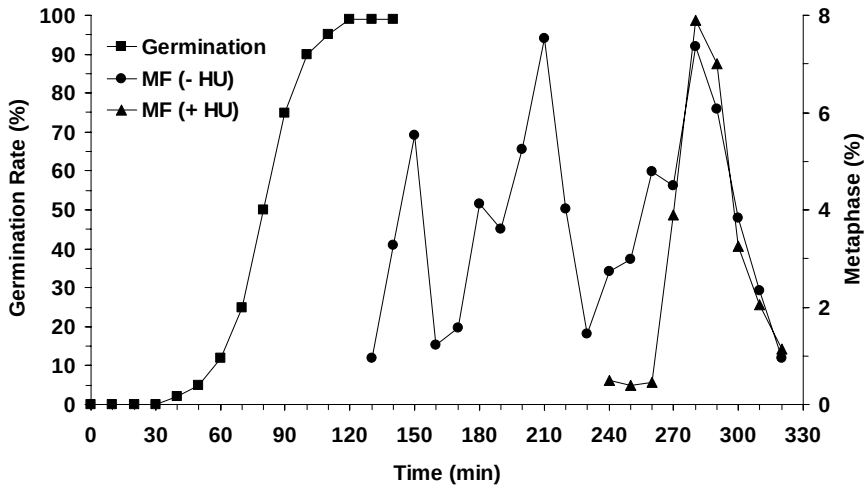

Figure 3.1. Time course evaluation of germination rate and metaphase frequency with and without hydroxyurea treatment on *Pyrenophora teres* conidia. Results for isolate WRS 1607 are shown. MF: metaphase frequency, HU: hydroxyurea.

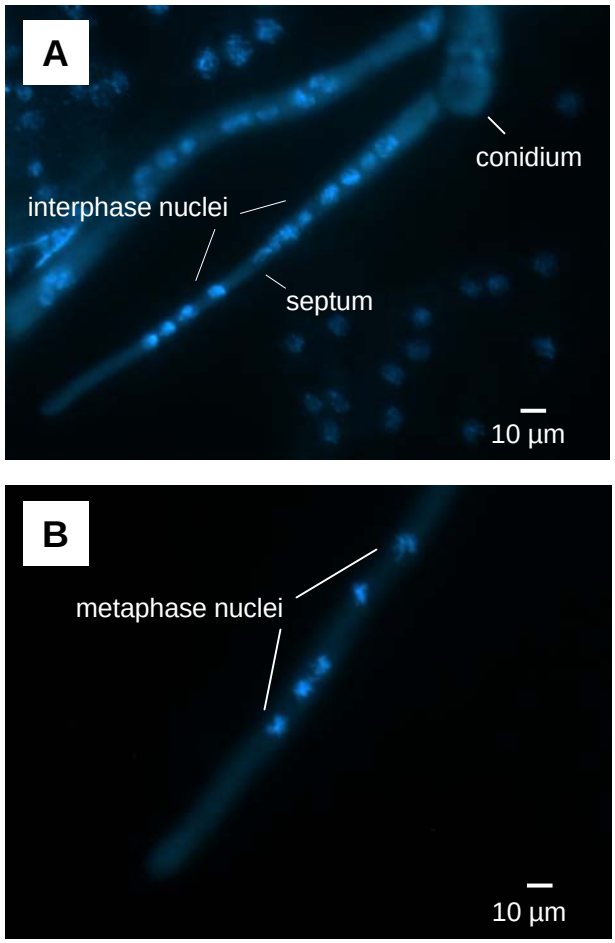

Figure 3.2. DAPI staining of synchronously dividing *Pyrenophora teres* nuclei. (A) Germ tube showing nuclei in interphase. (B) Metaphase stage nuclei.

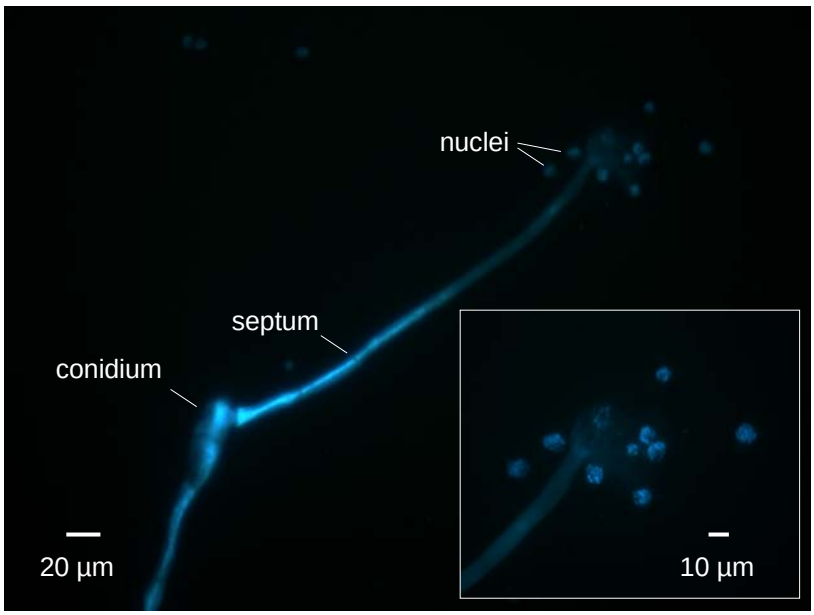

Figure 3.3. Example of nuclei discharged from the end of a *Pyrenophora teres* conidial germ tube burst using the GTBM. Inset is an enlargement of the germ tube end.

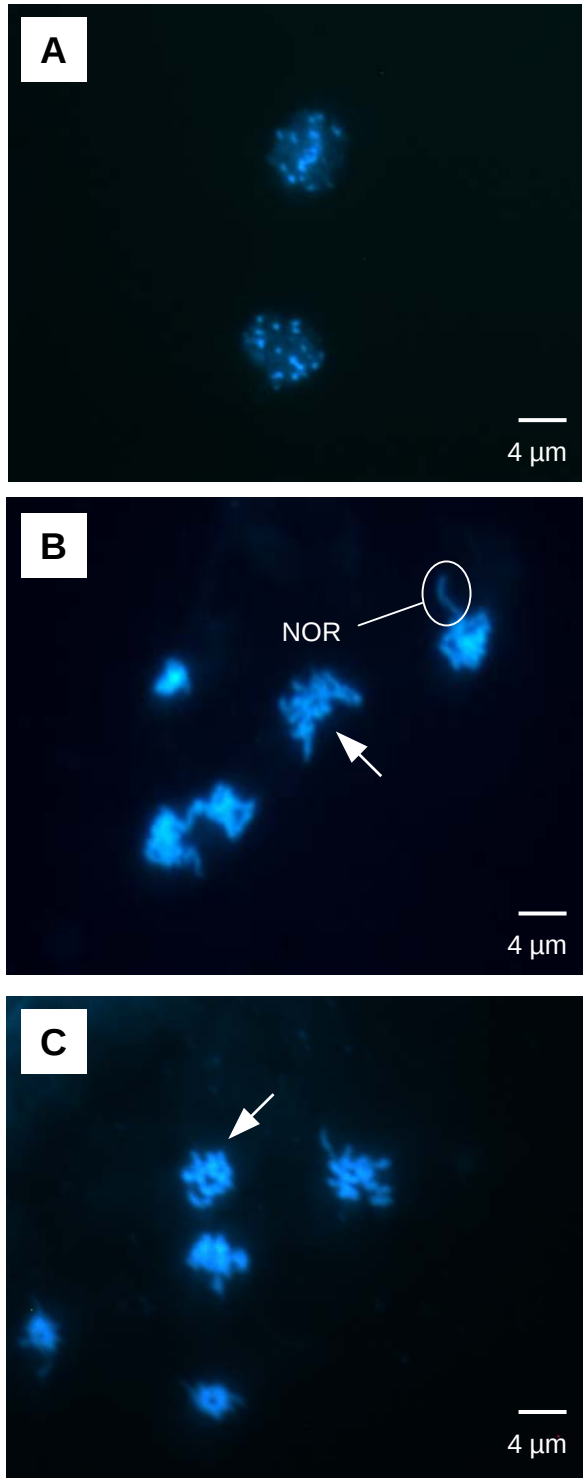

Figure 3.4. The GTBM and various discharged nuclei and chromosomes from *Pyrenophora teres*. (A) Nucleus. The brightly flourescent spots are constitutive heterochromatin. (B) Moderately condensed chromosomes likely in early metaphase (indicated by arrow). NOR: nucleolar organizer region. (C) Highly condensed metaphase chromosomes (indicated by arrow).

observed in interphase nuclei (Figure 3.4A), as were thin, elongated threads (the nucleolar organizer region) attached to individual chromosomes from metaphase or early metaphase nuclei (Figure 3.4B). Condensed metaphase chromosome spreads were detected for all isolates (Figure 3.4C) and allowed analysis of chromosome number. Nine chromosomes were observed for all *P. teres* isolates (Figure 3.5). Chromosome lengths ranged from 0.5 to 3.0 µm in length. PFGE analysis revealed eight bands for isolates SK2, WRS 1906 and WRS 857, while six bands were observed for isolates WRS 1607 and SK4 (Figure 3.6). Analysis of the intensity and size of the bands indicated that some represented multiple chromosomes which resulted in a total count of nine chromosomes for each of the isolates (Table 3.2). Significant CLP were observed between several isolates. The chromosomes ranged from 1.8 to ~6.0 Mb and provided a total genome size estimate of 34-37 Mb (Table 3.2).

Table 3.2. Chromosome number, sizes and total genome length estimated from PFG data for net and spot form *Pyrenophora teres* isolates.

Form	Isolate	Band	Vol.[1]	Chrom.[2]	Size(s)[3]
Net	WRS 1906	A	17300	2	6.0
		B	8800	1	5.1
		C	8900	1	4.6
		D	9200	1	3.9
		E	9600	1	3.0
		F	8800	1	2.9
		G	8000	1	2.5
		H	8000	1	2.2
			Total:	9	36.2
	WRS 1607	A	25000	2	6.0
		B	22400	2	4.7
		C	11400	1	4.1
		D	23800	2	3.5
		E	13300	1	2.7
		F	13800	1	1.8
			Total:	9	37.0
	SK2	A	11200	1	6.0
		B	8800	1	5.1
		C	10100	1	4.6
		D	18900	2	3.9
		E	10900	1	3.0
		F	8600	1	2.9
		G	9200	1	2.6
		H	9300	1	2.3
			Total:	9	34.3
Spot	WRS 857	A	21200	2	6.0
		B	9600	1	5.1
		C	11300	1	4.6
		D	10000	1	3.9
		E	12000	1	3.0

Table 3.2. (continued).

Form	Isolate	Band	Vol.[1]	Chrom.[2]	Size(s)[3]
		F	11400	1	2.9
		G	10700	1	2.5
		H	10000	1	2.2
			Total:	9	36.2
	SK4	A	23700	2	6.0
		B	20600	2	4.7
		C	12800	1	4.1
		D	25800	2	3.5
		E	13000	1	2.7
		F	10500	1	1.8
			Total:	9	37.0

[1] Band volume = intensity × area
[2] Estimated number of chromosomes per band
[3] Chromosome size expressed in Mb

Discussion

This study provides the first karyotype of both the net and spot forms of *P. teres* using the GTBM in combination with PFGE. Prior to this, information on the *P. teres* genome was extremely limited. Only one previous study had examined the karyotype of this pathogen. Aragona et al. (2000) used PFGE to study a single Sardinian isolate (net or spot form not stated), but the estimate of chromosome number was hindered by clustering of several chromosomes to a single band, a common problem with PFGE. Clustering was also observed in the current study where several PFG bands appeared to contain multiple chromosomes. However, chromosome counts obtained with the GTBM, in combination with densitometry analysis, helped resolve the likely number of chromosomes per band.

Nine chromosomes were observed using the GTBM for all isolates representing both forms of the pathogen, although counts ranging from eight to ten were recorded. Variable counts were also noted by Tsuchiya and Taga (2001) during analysis of three *Cochliobolus* species with the GTBM, thus this phenomenon appears to be normal with this method. However, evaluating multiple chromosome spreads with the GTBM allows confident assessment of karyotype by adopting the most frequent count as the true chromosome complement.

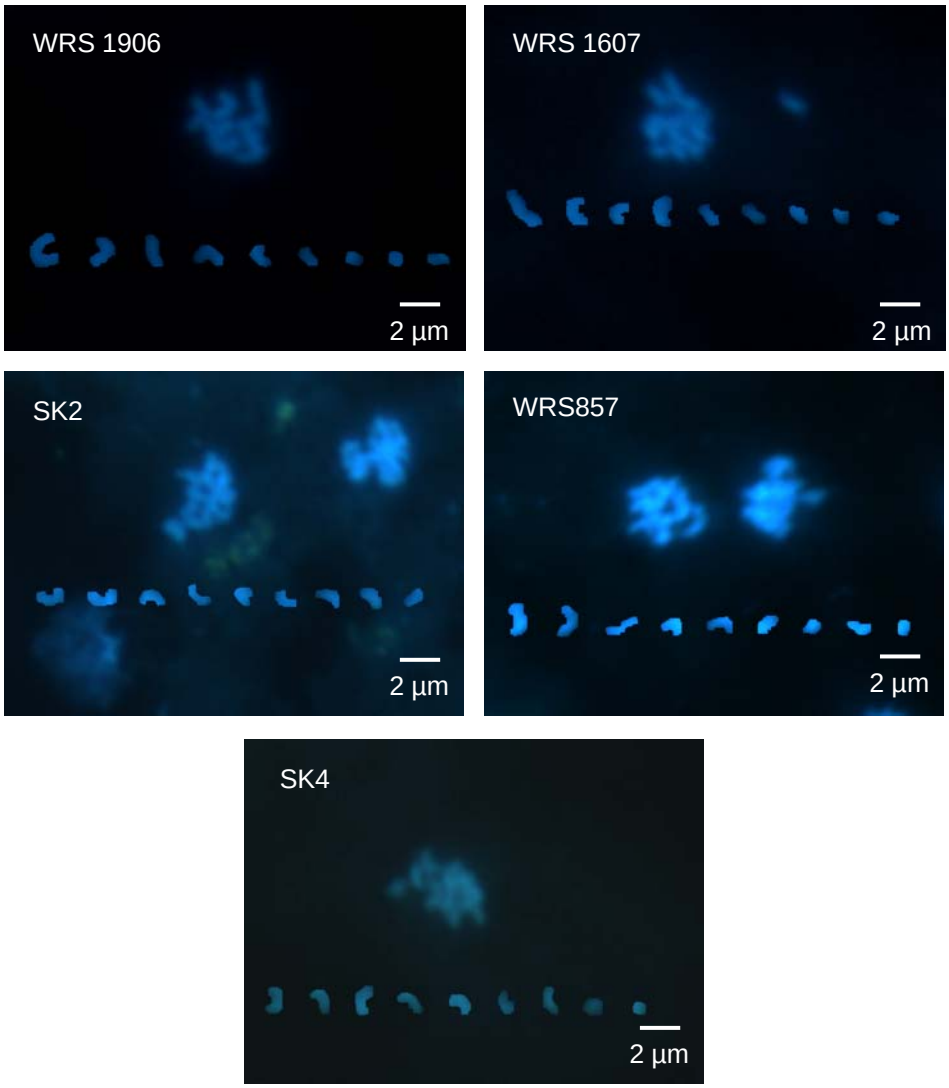

Figure 3.5. Karyotype analysis of the *Pyrenophora teres* net and spot form isolates used in this study. Nine chromosomes were counted in all isolates.

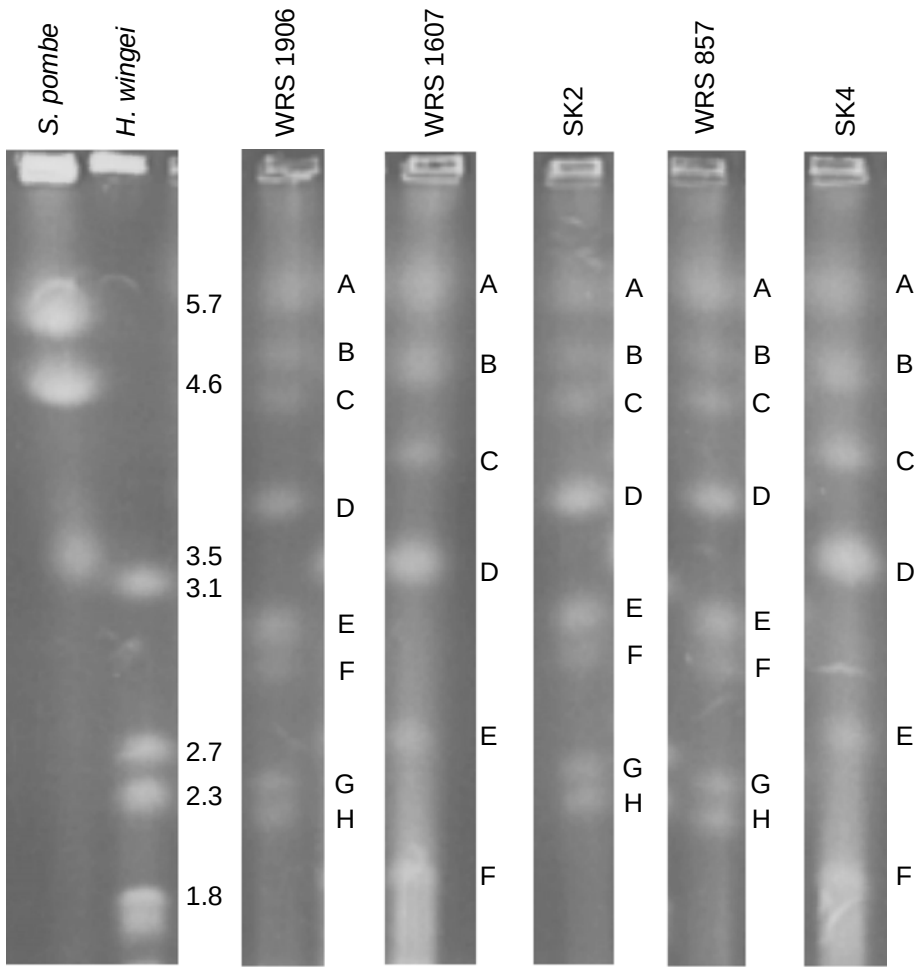

Figure 3.6. PFG-separated chromosomes of the *Pyrenophora teres* net and spot form isolates used in this study. The chromosomes of *Schizosaccharomyces pombe* and *Hansenula wingei* were used as size standards and sizes are shown in megabase pairs. Chromosomal bands for the isolates are indicated by letters to the right of each panel.

PFGE allowed an estimation of total genome size of approximately 34-37 Mb for both the net and spot form isolates of *P. teres*. This estimate falls within the range of genome sizes (10-40 Mb) typical for fungal species (Beadle et al. 2003). Chromosome-length polymorphisms (CLP) were also evident between *P. teres* isolates, a common feature in fungal genomes. Extensive variation has been observed in a large number of species including *Alternaria alternata* (Akamatsu et al. 1999), *Cochliobolus sativus* (Zhong et al. 2002) and *Pyrenophora tritici-repentis* (Lichter et al. 2002). A variety of chromosomal events can lead to CLP and repetitive DNA plays a significant role in these processes by acting as an initiation point for reciprocal translocations, deletions, inversions or duplications within a chromosome (Fierro and Martin 1999). Hybridization of several dozen AFLP markers to the chromosomes of WRS 1906 and WRS 1607 indicated that the *P. teres* genome contains a significant proportion of repetitive DNA since strong binding to multiple chromosomes was commonly observed (data not shown). It is likely that this repetitive DNA would be an important mechanism for CLP generation in *P. teres*.

The significant CLP observed among the net form isolates appear to have no effect on their ability to mate. Successful crosses which produced abundant progeny have been made between WRS 1607 and both SK2 and WRS 1906 (data not shown). Kistler and Miao (1992) suggested that the occurrence of CLP would be inversely proportional to the frequency of meiosis as significant changes in the genome, such as translocations, would decrease the fertility of crosses. However, work with *Leptosphaeria maculans* (Plummer and Howlett 1993), *Septoria tritici* (McDonald and Martinez 1991) and this study indicate that significant CLP can exist in sexual fungi without affecting fertility. In fact, analysis of tetrads has demonstrated that CLP can arise during meiosis to produce novel chromosomes differing in size from either parent (Gaudet et al. 1998; Plummer and Howlett 1993).

The differences in disease symptoms produced by the net and spot form isolates of *P. teres* are not reflected in large scale genomic changes, as indicated by the similar karyotypes of WRS 1906 (net form) and WRS 857 (spot form) and also WRS 1607 (net

form) and SK4 (spot form) isolates. Previous work with a population of isolates derived from a net form by spot form cross demonstrated that the production of spot versus net symptoms is controlled by two independent loci (one controlling each symptom) (Smedegard-Petersen 1977b). These differences have not resulted in discrete races or pathogenic groups based on form. This situation contrasts that of the HST-producing phytopathogens, where significant changes in host pathogenicity are often associated with single loci and large genomic changes, such as the acquisition of supernumerary chromosomes or reciprocal translocations. For example, the AK-toxin of *A. alternata*, which confers pathogenicity on Japanese pear, is located on a supernumery 1.05 Mb chromosome in pathogenic isolates (Hatta et al. 2002) and the *Tox1* gene of *C. heterostrophus*, present in isolates pathogenic on Texas male sterile corn, is thought to have been generated from a reciprocal translocation between chromosomes 6 and 12 (Tzeng et al. 1992).

The more closely related tan spot pathogen of wheat (*Pyrenophora tritici-repentis*), which also presents variable disease symptoms, provides some helpful insights into the relationship between pathogenicity and genome polymorphism that better reflects the relationship between *P. teres* and barley. This pathogen produces three toxins (HSTs) which are not only responsible for eliciting the chlorotic and necrotic symptoms typical of this pathogen, but also partition the isolates into distinct races (Strelkov and Lamari 2003). This pathogen follows a modified toxin model where toxin production mediates compatible interactions with genotypes of a single host species, as opposed to interactions with an entire host species. As such, it represents a type of interaction with the host which is intermediate between the HST pathogens which can colonize an entirely new host based on the acquisition of a single toxin, and the specific gene-for-gene interactions observed with specialized pathogens like biotrophic rusts and powdery mildew fungi. In an attempt to discern if a supernumerary chromosome was responsible for the differentiation of isolates carrying one such HST (ToxA), Lichter et al. (2002) found the *ToxA* gene was located on a 3.0 Mb chromosome from pathogenic isolates. However, a 2.75 Mb chromosome in non-pathogenic isolates was determined to be the equivalent chromosome since most probes derived from the 3.0 Mb chromosome

hybridized to the 2.75 Mb chromosome. They concluded that no single, simple event (ie. a supernumerary chromosome) was responsible for the difference in pathogenicity observed between the two types of isolates.

This study has provided new insights into the *P. teres* genome. It has revealed that the chromosome number in the two forms is identical, but that CLP exist between isolates. However, no CLP could be associated with the differences observed in disease symptoms between the two forms. An accurate assessment of the *P. teres* chromosome complement using the GTBM will assist genetic mapping by providing a target number of linkage groups that should be detected, while PFG separated chromosomes will help in the assignment of linkage groups to chromosomes by hybridization of markers to the PFG bands.

Chapter 4: A Genetic Linkage Map of *Pyrenophora teres* f. *teres*

Abstract

A genetic linkage map of the barley net blotch pathogen, *Pyrenophora teres* f. *teres*, was constructed using a population of 67 progeny derived from the cross between isolates WRS 1906 × WRS 1607. The map consists of 138 markers including 114 AFLPs, 21 telomere RFLPs, the mating-type (*MAT*) locus and an avirulence locus ($Avr_{Heartland}$) controlling interaction of isolate WRS 1906 with the barley cultivar 'Heartland.' Markers were distributed across 24 linkage groups ranging in length from 2 to 110 cM with an average marker interval of 8.5 cM. Total map length was 797 cM. A telomere-specific probe, $(TTAGGC)_4$, was used to map 15 of 18 telomeres. One of these telomeres mapped to within 3 cM of the $Avr_{Heartland}$ locus. Attempts to consolidate linkage groups by hybridizing markers to the electrophoretically separated chromosomes was unsuccessful because probes bound to multiple chromosomes, likely due to repetitive DNA within probes. This is the first genetic map reported for this species and it will be a useful genetic tool for map-based cloning of the $Avr_{Heartland}$ gene tagged in this study.

Introduction

Genetic maps establish basic information about genome organization and provide a foundation for genomic studies by creating a framework of ordered markers that can be exploited as reference points. Maps created in a number of important plant pathogenic fungi and oomycetes such as *Magnaporthe grisea* (Nitta et al. 1997), *Phytophthora infestans* (Van der Lee et al. 1997) and *Fusarium graminearum* (Gale et al. 2005) have been used to order genomic libraries (Martin et al. 2002), position genes relative to one another (Van der Lee et al. 2001), assist map-based cloning (Orbach et al. 2000) and validate sequence assembly (Gale et al. 2005).

Mapping avirulence (*Avr*) genes is one of the most common applications of fungal and oomycete genetic maps. These genes are found in bacterial, fungal, oomycete, viral and insect pathogens and mediate specific gene-for-gene interactions with resistance genes in the plant host as proposed by Flor (1955). Avirulence genes have been mapped in a number of phytopathogens including *Mycosphaerella graminicola* (Kema et al. 2002), *Leptosphaeria maculans* (Cozijnsen et al. 2000), *P. sojae* (Whisson et al. (1995) and *Magnaporthe grisea* (Dioh et al. 2000). Avirulence genes cloned from bacterial pathogens of *Arabidopsis* provide most of the knowledge about these genes, however, most plant pathogens are fungal or oomycete species so a greater understanding of *Avr* genes from them is warranted. There are currently nine *Avr* genes isolated from fungal and oomycete species: A*vr9* (Van Kan et al. 1991), *Avr4* (Joosten et al. 1994) and *Avr2* (Luderer et al. 2002) from *Cladosporium fulvum*, *Nip1* from *Rhynchosporium secalis* (Rohe et al. 1995), *AVR-Pita* from *Magnaporthe grisea* (Orbach et al. 2000), *AvrL567* from *Melampsora lini* (Dodds et al. 2004), *Avr1b-1* from *Phytophthora sojae* (Shan et al. 2004) and *ATR1*^{NaWsB} (Rehmany et al. 2005) and *ATR13* (Allen et al. 2004) from *Peronospora parasitica*.

Pyrenophora teres f. *teres*, causative agent of barley net blotch, is an economically important pathogen throughout the barley growing regions of the world. Yield losses ranging from 15-35% (Khan 1987; Martin 1985; Steffenson et al. 1991) and reduced grain quality (Mathre 1997) result in significant financial losses. Despite the importance of this pathogen, it remains unclear how *P. teres* interacts with the barley host. Identification of a locus that controls interaction with a specific barley genotype led to the suggestion that *P. teres* follows a cultivar specific toxin model (Weiland et al. 1999), similar to that observed with the related tan spot pathogen of wheat, *P. tritici-repentis*. In the *P. tritici-repentis* system, three toxins allow differentiation of eight races based on variable expression of necrotic or chlorotic symptoms on three wheat differential lines (Strelkov and Lamari 2003). However, the recent identification of a second *Avr* locus (this thesis), the isolation of only one toxin from *P. teres* (Friis et al. 1991) and the

numerous pathotypes that have been identified (Steffenson and Webster 1992; Tekauz 1990) suggest a gene-for-gene interaction for *P. teres*.

This paper reports on the construction of the first genetic map for *P. teres* f. *teres*. The map includes the location of the mating-type (*MAT*) locus, the $Avr_{Heartland}$ gene and several telomeres. This map will be a valuable tool to facilitate map-based cloning of *Avr* genes and improve our understanding of the interaction between *P. teres* and barley.

Materials and Methods

Pyrenophora teres Parental Isolates

The *P. teres* f. *teres* isolates WRS 1906 and WRS 1607 were selected as parents to create the mapping population based on previous knowledge (Chapter 2) that they are sexually compatible and display differential virulence on the barley cultivar 'Heartland' (Table 4.1). This cross allowed mapping of the mating-type locus and the avirulence gene controlling incompatibility on Heartland. Both isolates were highly virulent on the barley cultivar 'Harrington' (Table 4.1) which was used as a positive control during virulence phenotyping.

Table 4.1. Description of parental *Pyrenophora teres* isolates used to create the mapping population.

		Collection		Disease Reaction[2]		Mating-
Isolate	Form	Date	Location	Harrington	Heartland	Type
WRS 1906[1]	net	1994	Fredricton, NB	8	1	MAT-2
WRS 1607	net	1985	Prince Albert, SK	9	8	MAT-1

[1] WRS isolates provided by A. Tekauz (Cereal Research Centre, AAFC)

[2] Rated on a 1-10 scale (Tekauz 1985). Isolates which produce a rating of ≥6 are considered virulent while isolates eliciting a reaction of ≤5 are considered avirulent.

Pyrenophora teres Mating

Mating was carried out as described in Chapter 2.

DNA Extraction

DNA was isolated as described in Chapter 2.

AFLP Analysis

AFLP was carried out as described in Chapter 2 on the WRS 1906 × WRS 1607 mapping population. The 23 primer pairs identified in Chapter 2 which showed a high number of scorable polymorphic bands were used to screen the population.

Telomere RFLP Analysis

Genomic DNA (1.5 µg) was digested with *Eco*RI, *Eco*RV, *Hind*III, *Pvu*II and *Xho*I for 4 h. Digests contained 0.33 µg/µL RNaseA. Digests were loaded on 0.8% agarose gels and electrophoresed in 0.5× TBE for 15 h at 32 V. Gels were stained with 0.5 µg/mL ethidium bromide and photographed under UV light. For Southern blotting, gels were acid nicked in 0.25 M HCl for 15 min, denatured in 1.5 M NaCl, 0.5 M NaOH for 30 min and neutralized in 1.5 M NaCl, 0.5 M Tris-Cl (pH 7.5) for 30 min. DNA was transferred to Hybond N+ (Amersham Biosciences Inc., Baie d'Urfe, QC) in 20× SSC for 21 h, left to dry for 90 min and then covalently linked at 254 nm using a UV crosslinker (Ultra-Lum Inc., Claremont, CA).

Membranes were soaked in 2× SSC before being prehybridized for 2 h at 47°C in 1% dextran sulfate, 0.6 M NaCl, 4mM EDTA (pH 8.0), 120 mM Tris-Cl (pH 8.0), 2.2 mM tetra-sodium pyrophosphate, 10× Denhardt's reagent, 0.1% SDS and 50 µg/mL denatured Herring Sperm DNA. The oligonucleotide $(TTAGGG)_4$, corresponding to the conserved telomere repeat sequence, was end-labelled and hybridized to the DNA membranes. Labelling reactions were carried out according to the manufacturer's directions and included 25 ng oligo, 1 U T4 PNK (Invitrogen, Carlsbad, CA) and 25 µCi [γ-^{32}P]ATP (6000 Ci/mmol- Amersham Biosciences). Probes were purified through a G-50 spin column (Amersham Biosciences). Hybridization took place at 47°C overnight

in the same solution as prehybridizations except the dextran sulfate concentration was increased to 10%. Membranes were washed briefly in 2× SSC, 0.1% SDS at 47°C, then for 15 min at 50°C, followed by a final wash at 55°C for 15 min. Membranes were exposed to BioMax MR film (Eastman Kodak, Rochester, NY) using a BioMax HE intensifying screen (Eastman Kodak) at -80°C.

Markers were named using the first letter of the restriction enzyme and the band size. For example, the marker X1800 denotes a band 1800 bp in length produced with the *Xho*I restriction enzyme. EI and EV were used to distinguish *Eco*RI from *Eco*RV.

Bal31 Nuclease Assay

Approximately 3 µg of genomic DNA was digested with 4 U of Bal31 (Invitrogen) at 30°C according to the manufacturer's instructions and aliquots were removed at 0, 1, 2, 5, 10, 20 and 30 min. Reactions were stopped by adding $1/10^{th}$ volume of 0.5 M EDTA (pH 8.0). DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitated. Aliquots were digested with *Hind*III, electrophoresed and transferred to Hybond N+ as described previously. The membrane was hybridized with the telomere probe as described above. After stripping the membrane, a non-telomeric probe (the *MAT-1* allele) was hybridized to the membrane.

Mating-Type Analysis

Mating-type of the mapping population isolates was determined using PCR primers specific to the *MAT-1* and *MAT-2* alleles (Rau et al. 2005) as described in Chapter 2.

Data Analysis and Map Construction

All AFLP, telomere RFLPs and mating-type markers, along with the virulence phenotypic data obtained in Chapter 2 on the WRS 1906 × WRS 1607 population, were tested for deviations from the expected 1:1 segregation ratio using the χ^2 test ($\alpha = 0.05$).

For the purposes of mapping, virulence ratings similar to WRS 1906 (ie. low virulence) and markers originating from this parent were scored as 'a' while virulence ratings similar to WRS 1607 (ie. high virulence) and markers originating from this parent were scored as 'b.'

JoinMap 3.0 (Van Ooijen and Voorrips 2001) was used to group and order the markers and virulence phenotype data. Markers showing segregation distortion at ≥5% significance level were removed from the analysis. Initial linkage groups were established at several LOD scores (ranging from 2.0 to 5.0). A LOD of 4.0 was determined to be a suitable significance level because groups did not change at higher LOD scores. Linkage groups were then ordered (chi-square jump restriction = 5, maximum recombination value = 0.5, minimum LOD = 1.0) and a goodness-of-fit (ripple) performed after the addition of each new marker to the growing linkage group. Linkage between ordered loci was calculated using the Kosambi mapping function (Kosambi 1944).

Protoplast Isolation and Pulsed Field Gel Electrophoresis

Isolation of protoplasts from the parental isolates and pulsed field gel electrophoresis (PFGE) conditions were described in Chapter 3.

Hybridization of AFLP Markers to *Pyrenophora teres* Chromosomes

Two AFLP markers from each of the linkage groups defined by JoinMap were selected for hybridization to the *P. teres* chromosomes. AFLP bands were removed with a scalpel from silver stained 6% polyacrylamide gels, placed in a microfuge tube containing 10 µL distilled water and allowed to diffuse into the water overnight at 4°C. A 1 µL aliquot from each tube was reamplified using the same AFLP primers and selective amplification PCR conditions used to originally generate the marker. Reamplified markers were checked for correct band size on 6% polyacrylamide gels.

Reamplified AFLP markers were labelled by the random hexamer method according to the manufacturer's directions and included 25 ng DNA probe, 3 U Klenow (Invitrogen) and 30 μCi [α-^{32}P]dCTP (6000 Ci/mmol- Amersham Biosciences). Membranes were prehybridized for 4 h at 65°C and hybridized at 65°C overnight in the same solutions noted above. Membranes were rinsed briefly in 2× SSC, 0.1% SDS at 65°C, then washed for 20 min at 65°C and exposed to film as described above.

Results

Marker Analysis

The 23 AFLP primer pairs selected in Chapter 2 produced a total of 132 polymorphic markers. When the markers were screened across the entire mapping population, 124 (94 %) segregated in a 1:1 Mendelian ratio (Figure 4.1). The mating-type PCR primers determined that 35 of the progeny contained the *MAT-1* allele while 32 contained the *MAT-2* allele (χ^2 = 0.059, P = 0.807). This also fit a 1:1 ratio and indicated a single gene controlled mating-type. No isolate contained both alleles.

Telomere Analysis

Restriction digests of parental DNA produced numerous bands to which the telomeric probe, $(TTAGGG)_4$, could hybridize. Between 6 and 18 telomeric bands were identified with each of the restriction enzymes used (Figure 4.2). Very few common bands were observed between the parents in any of the digests, indicating a high level of polymorphism at the chromosome ends. A total of 30 polymorphic bands were identified for screening across the population, of which 26 (87 %) segregated in a 1:1 ratio.

All of the *Hind*III bands to which the telomere probe bound showed sensitivity to Bal-31 nuclease (Figure 4.3A). Hybridization to most bands was abolished within 5 min.

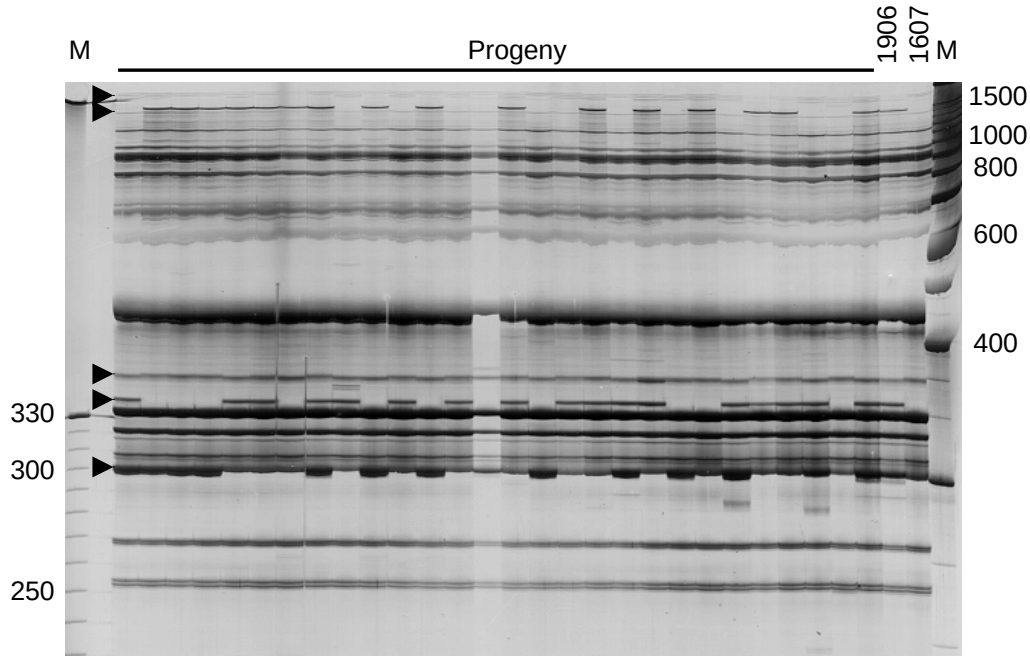

Figure 4.1. Example of an AFLP fingerprint generated in the *Pyrenophora teres* mapping population with primer combination E-CG/M-CC. Markers segregating in the population are indicated with arrows. Molecular size standards are shown in base pairs. M: marker lane.

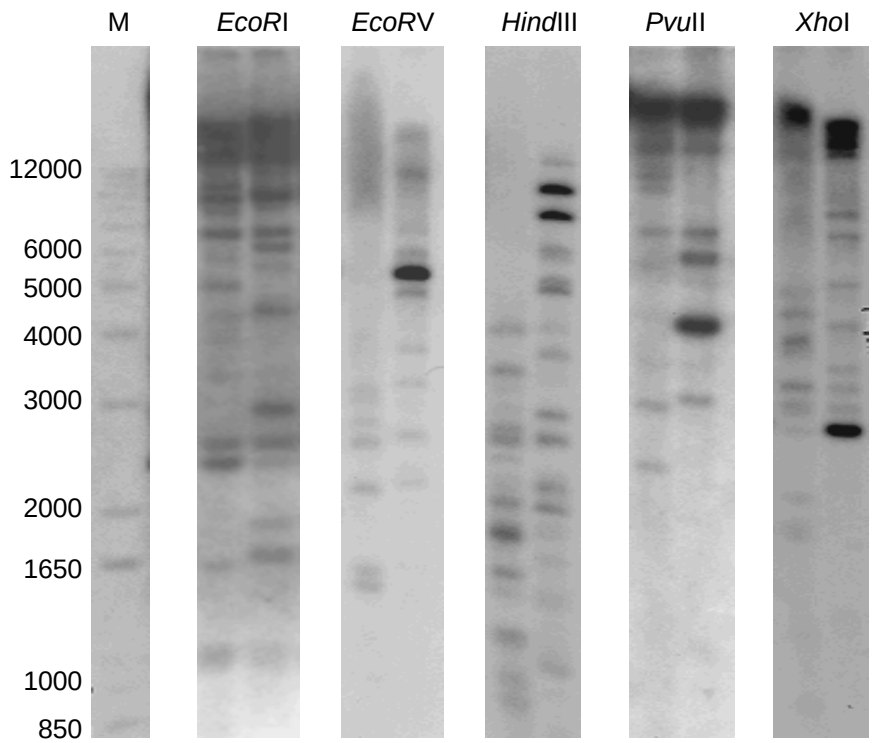

Figure 4.2. Telomeric RFLPs identified in the *Pyrenophora teres* parental isolates using the $(TTAGGG)_4$ probe. WRS 1906 genomic DNA is on the left and WRS 1607 DNA is on the right in each panel. Restriction enzymes used to generate the patterns are indicated at the top of each panel. Molecular size standards are shown in base pairs. M: marker lane.

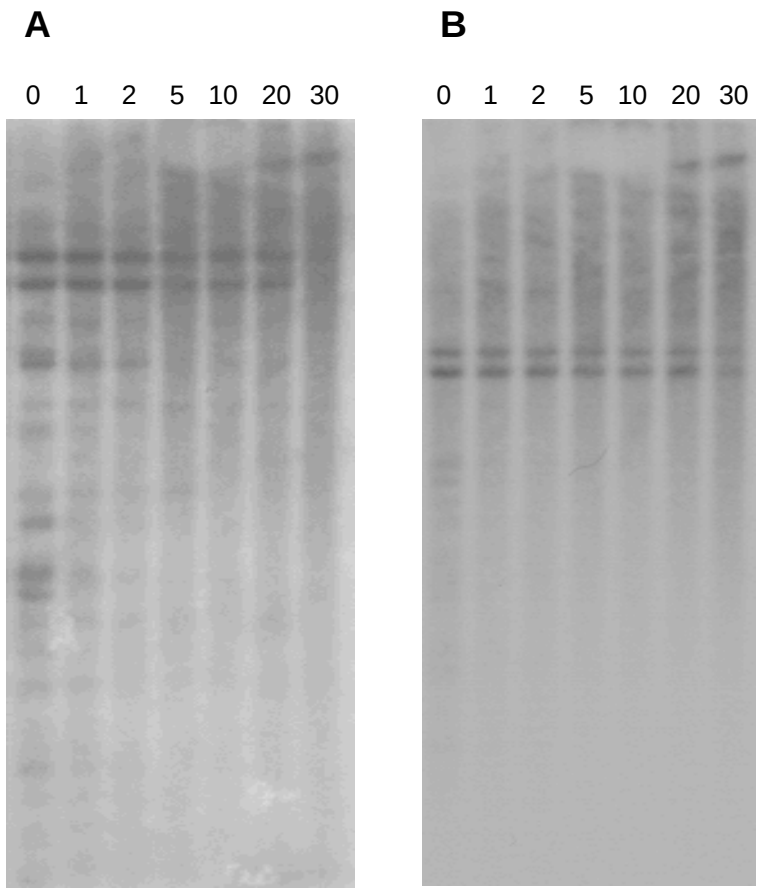

Figure 4.3. Southern blot analysis of *Pyrenophora teres* parental isolate WRS 1607 genomic DNA treated with Bal31 nuclease. DNA was digested with Bal31 for the times indicated at the top of each lane (min), followed by digestion with *Hind*III. The blot was hybridized with the telomere probe $(TTAGGG)_4$ (A), stripped and re-hybridized with a *MAT-1* probe as a control (B).

Estimation of telomere length based on the difference in band size between the control and the time point before disappearance of the band was not possible, however, based on a digestion rate of 20 bp/min/U (manufacturer's guide) the loss of bands after 5 min would indicate an average telomere size of approximately 100 bp. Hybridization to the two largest bands was abolished by 30 min, indicating a telomere size closer to 600 bp. These results showed the bands were located at the termini of chromosomes. By contrast, the band detected by the *MAT-1* probe was unaffected by Bal-31 digestion (Figure 4.3B). The *MAT-1* allele has been mapped to the middle of a linkage group in other mapping populations (O. Manninen, personal communication).

Linkage Map and Marker Hybridization to Chromosomes

The linkage map of *P. teres* consisted of 138 markers which included 114 AFLP markers, 21 telomere markers, the mating-type (*MAT*) locus and the avirulence locus ($Avr_{Heartland}$) (Figure 4.4). The map contained 24 linkage groups (LG) ranging in length from 2-110 cM with an average marker spacing of 8.5 cM and a maximum interval of 25 cM (Table 4.2). Total map length was 797 cM (Table 4.2). The $Avr_{Heartland}$ locus mapped within 3 cM of a telomere on LG4, the *MAT* locus mapped to the end of LG12 and 15 telomeres were mapped (Figure 4.4).

Assignment of linkage groups to individual chromosomes was unsuccessful. The AFLP markers selected from each linkage group hybridized to multiple PFGE-separated chromosomes making a definite identification of chromosome origin impossible (Figure 4.5A). This is likely due to the presence of repetitive DNA within each marker. By contrast, the *MAT-2* allele, known to represent a single locus in the genome of MAT-2 mating-type isolates, was assigned to one chromosome in the WRS 1906 parental isolate (Figure 4.5B).

◄ Figure 4.4. Genetic linkage map of *Pyrenophora teres* created from the cross WRS 1906 × WRS 1607. The map includes 138 markers (114 AFLP markers, 21 telomere markers, the *MAT* locus and the $Avr_{Heartland}$ locus) distributed over 24 linkage groups with a total map length of 797 cM. AFLP markers were named using the four extension letters from the selective primers and the band size while the telomere markers were named using the first letter of the restriction enzyme used to create the RFLP and the band size.

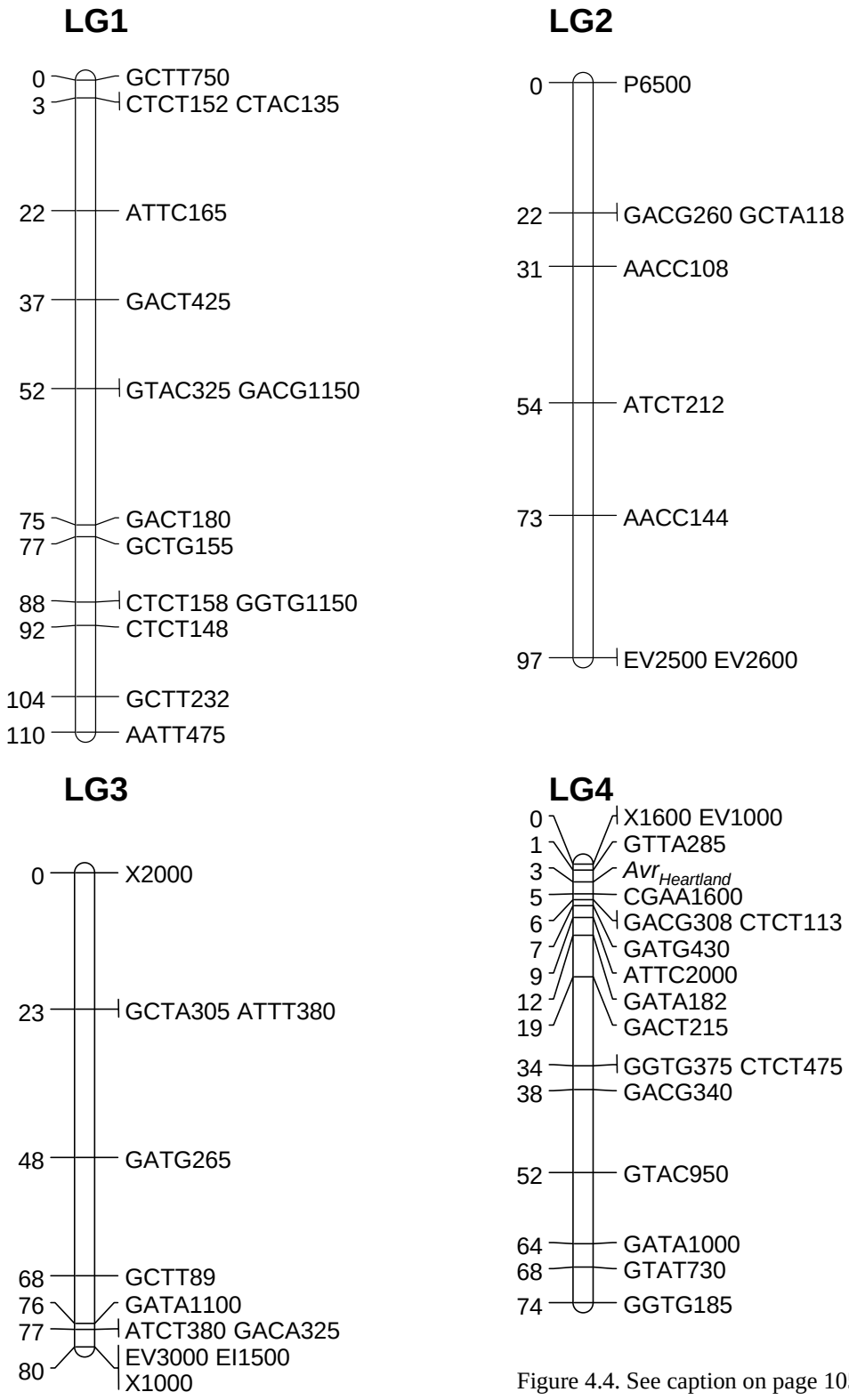

Figure 4.4. See caption on page 105.

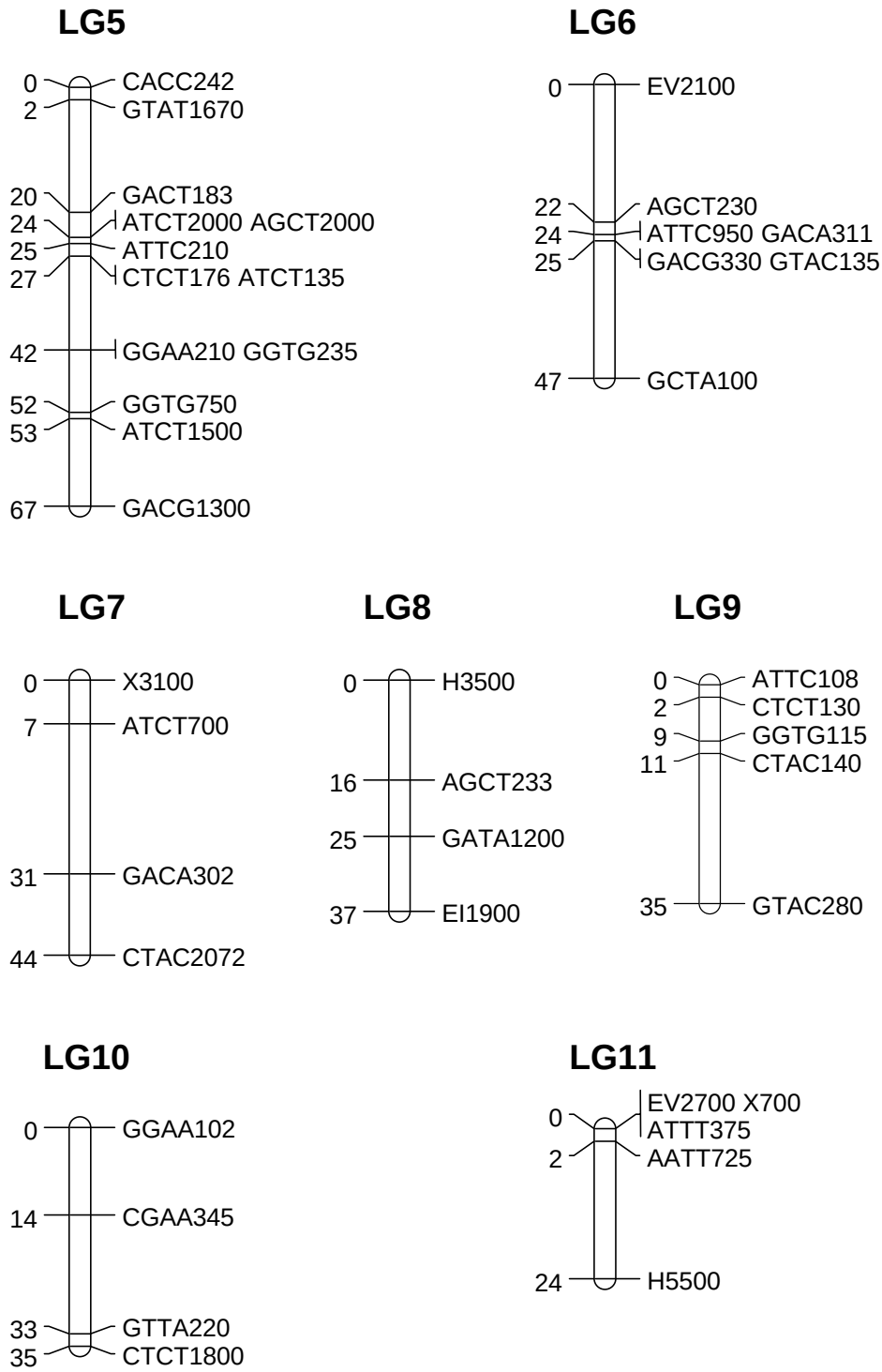

Figure 4.4. (continued).

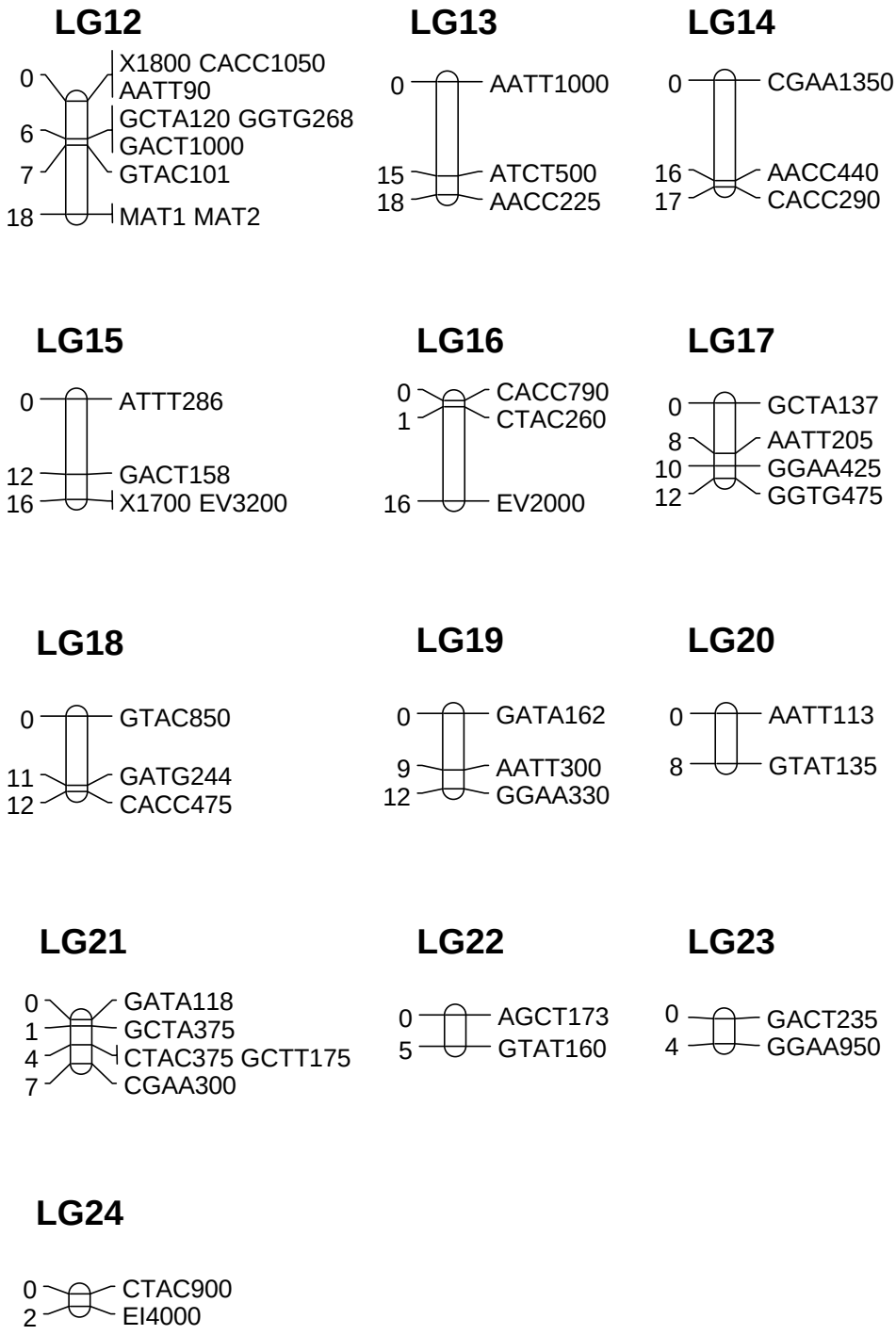

Figure 4.4. (continued).

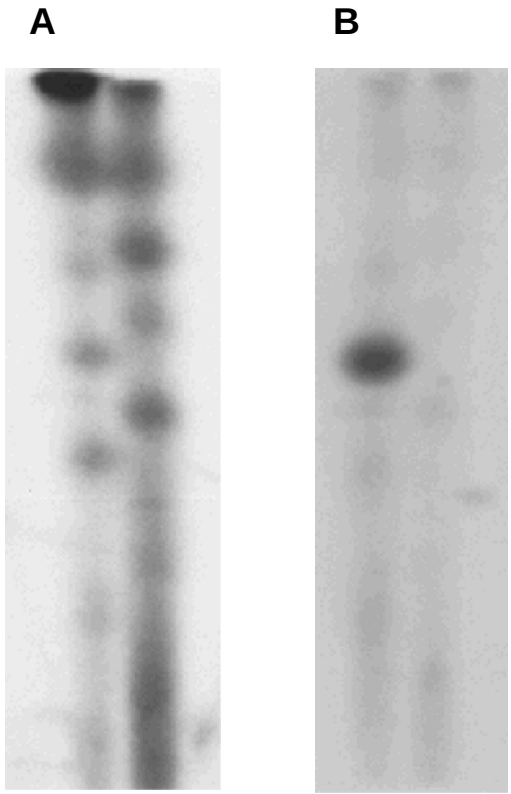

Figure 4.5. Southern blot of PFG-separated chromosomes from the *Pyrenophora teres* parental isolates WRS 1906 (left) and WRS 1607 (right). (A) Hybridization of AFLP marker GACG260 shows binding to multiple chromosomes in each isolate, typical of the AFLP probes used in this study. (B) Hybridization of the single copy *MAT-2* probe binds only to the 3.9 Mb chromosome of WRS 1906 (MAT-2 mating type).

Table 4.2. Description of *Pyrenophora teres* linkage groups.

Linkage Group	Number of Markers	Length (cM)	Average Interval (cM)	Largest Interval (cM)
1	14	110	11	23
2	8	97	19.4	24
3	11	80	13.3	25
4	18	74	5.3	15
5	13	67	7.4	18
6	7	47	11.8	22
7	4	44	14.7	24
8	4	37	12.3	16
9	5	35	8.8	24
10	4	35	11.7	19
11	5	24	12	22
12	9	18	6	11
13	3	18	9	15
14	3	17	8.5	16
15	4	16	8	12
16	3	16	8	15
17	4	12	4	8
18	3	12	6	11
19	3	12	6	9
20	2	8	8	8
21	5	7	2.3	3
22	2	5	5	5
23	2	4	4	4
24	2	2	2	2
Total:	138	797	8.5	

Discussion

Very few genetic linkage maps exist for phytopathogenic fungi and oomycetes. Maps currently exist for *Fusarium graminearum* (Gale et al. 2005; Jurgenson et al. 2002), *Cochliobolus heterostrophus* (Tzeng et al. 1992), *Phytophthora sojae* (Whisson et al. 1995), *P. infestans* (Van der Lee et al. 1997), *Cochliobolus sativus* (Zhong et al. 2002), *Leptosphaeria maculans* (Cozijnsen et al. 2000), *Mycosphaerella graminicola* (Kema et al. 2002) and *Magnaporthe grisea* (Nitta et al. 1997). The *P. teres* map created in this study is the first described for this species.

The *P. teres* linkage map is distributed across 24 linkage groups, which exceeds the nine chromosomes identified in this species (Chapter 3). The presence of more linkage groups than chromosomes is commonly observed with fungal and oomycete linkage

maps. For example, the *M. graminicola* map consists of 23 linkage groups, but only 17-18 chromosomes were observed with PFGE (Kema et al. 2002). Similarly, the *F. graminearum* chromosome complement is four, but both linkage maps for this pathogen contained nine linkage groups (Gale et al. 2005; Jurgenson et al. 2002).

The task of joining linkage groups belonging to the same chromosome into larger assemblies is made somewhat easier in fungal and oomycete species because their small chromosomes are amenable to PFGE and subsequent hybridization with marker probes. A number of maps integrating genetic linkage information with the physical genome have been produced in species such as, *M. grisea* (Sweigard et al. 1993), *C. heterostrophus* (Tzeng et al. 1992) and *C. sativus* (Zhong et al. 2002), and allow an estimate of genome coverage provided by the map. For example, the total map length of *C. heterostrophus* based on distance covered by linkage groups totalled 941 cM, but after assigning the linkage groups to chromosomes, 14 gaps of at least 40 cM (this was the maximum distance at which linkage could be established) were identified between the linkage groups producing a revised map length of 1501 cM (Tzeng et al. 1992).

Attempts to assign linkage groups to the *P. teres* chromosomes were unsuccessful because all of the AFLP markers hybridized to at least two chromosomes. This problem was also noted by Cozijnsen et al. (2000) who found approximately half of the AFLP markers that were used as probes bound to all *L. maculans* chromosomes. This is likely due to the presence of repetitive DNA sequence within the AFLP markers since many fungal genomes are known to contain a high proportion of such DNA. One possible solution to this problem would be to clone the AFLP markers and pre-screen for repetitive DNA by hybridization with total *P. teres* DNA. Under the assumption that clones containing little or no repetitive DNA would show low signal intensity, one could select clones that likely contain single copy DNA sequence.

Telomeres of many fungi, including *Podospora anserina*, *Neurospora crassa* and *Aspergillus nidulans*, contain the short repeating unit 5'-TTAGGG-3' (Bhattacharyya and Blackburn 1997; Javerzat et al. 1993; Schechtman 1990). Schechtman (1989) first

demonstrated that a probe consisting of this sequence could be used to map telomeres in *N. crassa*. This method was successfully applied by Farman and Leong (1995) to map the telomeres of all seven *M. grisea* chromosomes onto an existing linkage map. The map length increased from 620 cM to 922 cM demonstrating that a significant amount of the genome was not represented in the map. The current study is the first to report that *P. teres* telomeres are composed of this same hexamer sequence. Using this sequence as a probe, 15 of the 18 *P. teres* telomeres were mapped, including four linkage groups with telomeres mapped to both ends, presumably representing an entire chromosome. Despite mapping so many telomeres, a better estimate of genome coverage was not possible. The relationship between a large number of the linkage groups remains unknown, thus a substantial number of possible map configurations exist when attempting to place the groups together onto nine chromosomes.

It was interesting to note that some of the linkage groups associated with two telomeres were relatively small (eg. linkage group 8 at only 37 cM) while larger linkage groups such as number one (110 cM) showed no linkage to a telomere. However, genetic distance is not always a good indicator of physical distance. For example, linkage groups spanning approximately the same genetic distance (153-157 cM) were assigned to chromosomes 1 and 4 in *M. grisea* despite chromosome 1 being substantially physically larger than chromosome 4 (Farman and Leong 1995). Therefore, it may be that linkage group 1 in the *P. teres* map does not represent a very large physical distance and may be far from a telomere.

Another benefit of delimiting chromosome ends was demonstrated in *M. grisea* when map-based cloning of the *AVR-Pita Avr* gene was aided by the knowledge that this gene was tightly linked to a telomere (Orbach et al. 2000). Tight linkage between a telomere on linkage group 4 and the locus controlling avirulence of WRS 1906 on Heartland was also observed in this study. Analysis of the *AVR-Pita* gene in a number of *M. grisea* strains revealed a large number of mutations such as deletions, insertions and point mutations which abolished function of this gene (Orbach et al. 2000). It was hypothesized that this was a mechanism used by the pathogen to avoid detection by the

corresponding plant resistance gene. Similar mutations to the *Avr* gene identified in this study may account for the ability of isolates, such as WRS 1607, to infect Heartland.

This map of the *P. teres* pathogen will be a valuable tool for map-based cloning of genes, including the $Avr_{Heartland}$ locus identified in this study. Information derived from such genes will further our understanding of how and where the products of these genes function and the manner in which they are recognized by resistant barley plants. The addition of more markers and the use of larger mapping populations will help expand map coverage of the genome and consolidate the linkage groups, improving the utility of the map.

General Discussion

This research provides several new insights into the *P. teres* pathogen. Evidence provided indicates the *P. teres*-barley pathosystem follows the gene-for-gene model. This was shown by the presence of significant pathotype variability during virulence phenotyping and the identification of a locus ($Avr_{Heartland}$) which controls avirulence of isolate WRS 1906 on Heartland. The chromosome complement of both the net and spot forms was determined for the first time. Nine chromosomes were observed in all isolates along with a variety of CLP. These differences in genome structure were not a barrier to reproduction and could not account for the differences in disease symptoms observed between the net and spot forms. The first genetic linkage map for *P. teres* was created. It includes the locations of the $Avr_{Heartland}$ locus, the mating-type (*MAT*) gene and 15 of 18 telomeres.

Future work following from this thesis should be directed towards: 1) improving the current linkage map and, 2) cloning the $Avr_{Heartland}$ gene identified. Increasing the marker density of the linkage map created in this study would improve its usefulness by increasing the likelihood that markers will be tightly linked to genes of interest in future mapping studies. The use of DArT (Diversity Array Technology) would be one strategy to quickly create a well saturated map that could be integrated with the current map. This would likely bridge the gaps that presently exist between many of the linkage groups and reduce the total number to coincide with the chromosome complement of nine observed by the GTBM. The DArT array could also be used to quickly determine which clones on the array contain repetitive DNA by labelling total genomic DNA from the parental isolates and hybridizing this to the array. Clones showing low intensity signals could be used subsequently as probes for hybridization to PFG-separated chromosomes and permit assignment of linkage groups to chromosomes.

The linkage map has a number of potential applications other than gene mapping. Genetic maps created in *M. grisea* and *F. graminearum* have been used to assemble and

anchor BAC contigs and sequence data, respectively, to specific chromosomes (Gale et al. 2005; Martin et al. 2002). Such integrated maps allow rapid mapping of other traits (once they have been phenotyped) and association of these traits back to clones that harbour the responsible genes. A second application of genetic maps was demonstrated by Van der Lee et al. (2001). They used a pre-existing *P. infestans* map to integrate markers linked to six *Avr* genes (identified by BSA) and determine their relative position on the map, and noted that five of the six genes mapped to the most distal portions of the linkage groups. As a result, they proposed that future studies would determine if these genes were located close to the chromosome ends by mapping the telomeres. A final application of genetic maps involves assessing the effect of genome rearrangements. Because a map represents a set of ordered markers within the genome of the parental isolates, one can detect changes to this order in other isolates and determine any biological consequences. Large changes in genome structure are sometimes associated with altered pathogenicity, as observed with the HST-producing phytopathogens. For example, a reciprocal translocation between chromosomes 6 and 12 in *C. heterostrophus* is thought to have conferred pathogenicity on Texas male sterile corn (Tzeng et al. 1992).

The next step towards cloning the $Avr_{Heartland}$ gene identified in this study will require the creation of a large insert DNA library, such as a BAC, cosmid or λ library. Such libraries were essential for isolation of *Avr1b* from *P. infestans* (Shan et al. 2004), $ATR1^{NdWsB}$ and *ATR13* from *P. parasitica* (Allen et al. 2004; Rehmany et al. 2005), and *AvrL567* from *M. lini* (Dodds et al. 2004). In conjunction with a library, a larger mapping population should be phenotyped and screened to increase the number and proximity of markers around the $Avr_{Heartland}$ gene. This will provide useful landmarks to facilitate contig assembly during chromosome walking. For example, a mapping population of 311 *P. parasitica* isolates was screened with 273 AFLP primer pairs to refine the $ATR1^{NdWsB}$ interval to 1 cM (or 250 kb) (Rehmany et al. 2003) which assisted cloning this gene.

Cloning of *Avr* genes in other pathogens has allowed a variety of strategies to be pursued to isolate virulence targets and understand modes of action. Shao et al. (2003) used immunoprecipitation to determine AvrPphB from *P. syringae* interacts with *Arabidopsis* PBS1, required for RPS5-mediated resistance, and showed that AvrPphB triggers a resistance reaction upon cleaving the PBS1 protein. Chemical crosslinking of I^{125}-labelled AVR4 from *C. fulvum* demonstrated that it bound to a fungal-derived polysaccharide (Westerink et al. 2002), later shown to be chitin (Van den Burg et al. 2003), which indicated its role was to protect the invading mycelium from plant chitinases. The yeast-two-hybrid assay was used by Mackey et al. (2002) to identify *Arabidopsis* RIN4, the virulence target of AvrB from *P. syringae*, and demonstrated that RIN4 was phosphorylated by AvrB which activated a resistance reaction. They also showed that RIN4 bound to RPM1, the resistance protein in *Arabidopsis* that responds to the presence of AvrB, indicating this may be a possible method to identify the R protein partner of Avr proteins. Similarly, the *M. grisea* AVR-Pita protein and rice Pi-ta protein were demonstrated to bind to one another using the yeast-two-hybrid assay (Jia et al. 2000) after map-based cloning of each gene. Presumably, AVR-Pita could have been used as bait to isolate Pi-ta directly.

Cloning of another fungal *Avr* gene would provide insight into our limited knowledge of these genes, including their functions, how their products interact with plant *R* gene products and how they avoid them. Knowledge gained from the study of such *Avr*-*R* gene interactions would ultimately contribute to the goal of more durable resistance. Durable resistance has been rare since genetically uniform crops became common in modern agriculture. Some of the few examples include the *mlo* gene in barley which has been widely used in Europe to effectively control powdery mildew (Jergensen 1992), the *Rpg1* gene that has controlled barley stem rust for over 60 years (Brueggman et al. 2002) and the *Xa3* bacterial blight resistance gene from rice which has been used for 15 years (Bonman et al. 1992). Assessing the durability of resistance is a retrospective exercise that can only occur after growth of cultivars containing the *R* gene over many years and environments. However, several older ideas for achieving durable resistance are now being re-examined and supported with data from *R-Avr* studies.

The greatest genetic diversity for a given crop species can be found in wild populations where no disease epidemics occur despite high disease pressure (Leonard 1997). Several recent studies with *R* and *Avr* genes have demonstrated genetic diversity at the molecular level. Caicedo and Schaal (2004) observed that *Cf-2* alleles in wild tomato populations were highly variable, as was the *RCG2* locus in lettuce (Kuang et al. 2004). Evidence from other recently cloned *Avr* genes demonstrate that similarly high levels of diversity can be found in pathogen populations. For example, six highly divergent alleles of $ATR1^{NdWsB}$ from *P. parasitica* were identified from eight isolates (Rehmany et al. 2005). Such diversity is in contrast to modern agriculture where boom-and-bust cycles, as described with cereal rusts (Kolmer 1996) and powdery mildew (Brown et al. 1997), occur when one isolate flourishes as a result of selective pressure imposed by the use of single, major *R* genes (McDonald and Linde 2002). Observations of natural populations would suggest that a strategy of creating genetic diversity should be followed in order to control disease.

Following this line of reasoning, pyramiding *R* genes has been proposed as a means of attaining more durable resistance under the assumption that the pathogen will be unable to achieve multiple mutations that defeat several *R* genes. Singh et al. (2001) demonstrated that three rice bacterial blight *R* genes, *xa5*, *xa13* and *Xa21*, pyramided into a single plant by MAS could provide greater resistance against a wider spectrum of isolates than any of the individual genes alone. While demonstrating the benefits of such a strategy, this study also exemplified the large amount of prior work required to identify and understand the spectrum of isolates (*Avr* genes) that each *R* gene is effective against.

Another factor which must be considered when pyramiding *R* genes (and even when incorporating individual *R* genes) is the effect on plant fitness (yield) since the priorities of most breeding programs will place yield and quality characteristics above disease resistance. For example, the *ym4* gene that confers resistance to barley mild mosaic virus and strain 1 of barley yellow mosaic virus was associated with a 2% grain yield

loss (Le Gouis et al. 1999), however, it was unclear if the yield loss was due to the *R* gene alone or linkage drag. Stronger evidence that *R* genes can be responsible for yield depression was provided by Kolster et al. (1986). They evaluated doubled haploid barley progeny derived from crosses between three different *mlo* mutant lines and susceptible cultivars in trials where all foliar diseases were controlled (including powdery mildew). They saw a 4.2% yield reduction in *mlo* versus *Mlo* plants which was attributed to the *R* gene (and not linked genes) since similar yield costs were observed with each of the three *mlo* mutations in different cultivars. In some cases it appears that the cost of harbouring *R* genes can be too costly. Stahl et al. (1999) noted that *Rpm1* has been lost independently on many occasions from *Arabidopsis*, suggesting it may present a fitness cost that is too high in the absence of the corresponding isolate. This was confirmed by Tian et al. (2003) when *Arabidopsis* lines carrying *Rpm1* showed reduced fitness in the absence of the pathogen compared to lines lacking the gene.

A potential alternative to gene pyramiding is to create cultivar mixtures that are composed of several different *R* genes in a genetically uniform background which can be grown together or rotated on a yearly basis. Such a system would limit any deleterious fitness costs to a single *R* gene, while restricting the time a pathogen has to overcome a given *R* gene (in rotations) or the number of host plants should it overcome one of the *R* genes (in mixtures). In either system the goal is to keep the numbers of any given isolate at a given time as small as possible. Evidence that mixtures are effective at controlling disease was indicated in barley cultivar mixtures grown in Germany in the 1980s. As mixtures increase from 0 to 92% of seeded area, powdery mildew levels decreased from > 50% to < 10% with a corresponding three fold decrease in fungicide applications (Wolfe 1992). Similar reductions in disease levels were not observed in areas where mixtures were not grown. More recently Zhu et al. (2000) showed that rice blast levels on susceptible rice lines were reduced by 94% and yield increased by 89% when grown with resistant hybrids. They also noted that pathogen variability increased in the mixtures while only one or a few isolates dominated monoculture fields.

Efforts to isolate and evaluate *R-Avr* partners can make a valuable contribution to cultivar mixtures and gene pyramiding efforts by isolating and determining which *R* genes will prove to be the most durable prior to incorporation into cultivars. As Vera Cruz et al. (2000) demonstrated in their rice bacterial blight resistance study, evaluating the fitness cost associated with loss of an *Avr* gene (resulting from selective pressure imposed by an *R* gene) may be a good measure of *R* gene durability. That is, the larger the fitness cost associated with an *Avr* gene, the more durable the corresponding *R* gene is likely to be. This is certainly a concept which needs further exploration.

It has been observed that families of *Avr* genes, such as *avrBs3*, which encode functionally redundant proteins (Yang et al. 1996) may also impact on the selection of *R* genes for durable resistance. Such redundancy suggests the pathogen may more easily lose the function of one of these genes without suffering significant fitness loss. It would also be useful to determine the potential of an *Avr* gene to incur mutations which abolish R protein recognition but do not affect virulence function. As shown by Shan et al. (2000), several induced mutations in avrPto allowed *P. syringae* to avoid detection by Pto, but retain virulence. By conducting mutagenesis studies on more *Avr* genes it would be possible to identify those which do not show a separation of functions. For example, the Ry R protein from potato recognizes the Nla protein from potato virus Y. Nla is a protease and mutations to the active domain not only abolish its enzyme activity, but also Ry recognition (Mestre et al. 2000). This suggests that Ry will be a more durable *R* gene.

Finally, study of *Avr* genes corresponding to durable *R* genes such as *Rpg1*, *Lr34* and *Xa3* would be informative. Perhaps some common biological function among these *Avr* genes, or their virulence targets, may be revealed and could serve as a guide when searching for durable *R* genes against other pathogens.

There are still many questions and gaps in our knowledge pertaining to interactions between phytopathogens and their hosts despite recent significant progress. The large amount of data generated by the various "omic" approaches will hopefully address this

situation and provide insights that can be integrated with current breeding and agronomic approaches to disease control.

Conclusions

1. There is significant pathotype variability within Canadian *P. teres* isolates.
2. A single gene ($Avr_{Heartland}$) in *P. teres* f. *teres* isolate WRS 1906 is responsible for controlling avirulence on the barley line Heartland.
3. Points one and two provide evidence that the *P. teres*-barley pathosystem conforms to the gene-for-gene model.
4. There appears to be a large amount of repetitive DNA in the *P. teres* genome as indicated by the hybridization of AFLP markers to multiple chromosomes.
5. A total of nine chromosomes are present in both the net and spot form of *P. teres*.
6. Significant CLP exist among *P. teres* isolates, but none of these can account for the different disease symptoms elicited by the two forms.
7. CLP do not appear to be a reproductive barrier in *P. teres*.
8. A genetic linkage map of *P. teres* was produced that included 138 markers (including the mating-type (*MAT*) locus and $Avr_{Heartland}$ locus) distributed over 24 linkage groups and covered 797 cM. The map also includes 15 of the 18 telomeres, one of which is tightly linked to the $Avr_{Heartland}$ locus.

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Appendix A: Virulence Phenotypes and Association of AFLP Markers Linked to the Avirulence Locus for the WRS 1906 × WRS 1607 Population

Table A.1. Virulence phenotypes of the parental isolates, WRS 1906 and WRS 1607, and the 67 derived progeny on the barley differential line Heartland and association of AFLP markers linked to the avirulence locus (*Avr*$_{Heartland}$).

		AFLP Markers[2]						
Isolate	Virulence Rating[1]	GACT 215	GATA 182	GATG 430	GACG 308	CGAA 1600	GTTA 285	Bulk[3]
1	3 (2,4)	-	-	-	-	+	-	
2	8	+	+	+	+	-	+	V3
3	3 (4)	-	-	-	-	+	-	
4	8	+	+	+	+	-	+	
5	9 (8)	+	+	+	+	-	+	V1
6	3 (1)	-	-	-	-	+	-	
7	3 (2,4)	-	-	-	-	+	-	
8	2 (1)	-	-	-	-	+	+	A3
10	8 (7)	-	-	-	-	+	+	V3
35	8 (7)	+	+	+	+	-	+	V2
36	1 (2)	-	-	-	-	+	-	A3
39	9 (8)	+	+	+	+	-	+	V1
41	2 (5)	-	-	-	-	+	-	
42	2 (3)	-	-	+	-	+	-	
43	8 (9)	+	+	+	+	-	+	
44	8 (7,9)	-	-	-	-	+	+	V3
45	4 (5)	-	-	-	-	+	-	
46	8 (7,9)	+	+	+	+	-	+	V3
47	7 (6)	+	+	+	+	-	+	
48	2	-	-	-	-	+	-	
49	4 (5)	-	-	-	-	+	-	
50	9	+	+	+	+	-	+	V1
51	7 (6)	+	+	+	+	-	+	
52	2 (4)	-	-	-	-	+	-	
53	7	-	+	+	+	-	+	
54	8 (6)	+	+	+	+	-	+	
55	2 (1,4)	-	-	-	-	+	-	
56	2 (3)	+	+	-	-	+	-	
57	7 (6)	+	+	+	+	-	+	
58	2	-	-	-	-	+	-	
59	9 (8)	+	+	+	+	-	+	V2
60	1	-	-	-	-	+	-	A2
61	9	+		+	+	-	+	V1
62	2 (1)	+		-	-	+	-	A2
65	8 (7)	-		+	+	-	+	V3
66	1 (3)	+		-	-	+	-	A1
68	1 (2)	-		-	-	+	+	A2
70	4 (3)	-	-	-	-	+	-	A3
73	7 (6)	+	+	+	+	-	+	

Table A1. (continued).

		AFLP Markers[2]						
Isolate	Virulence Rating[1]	GACT 215	GATA 182	GATG 430	GACG 308	CGAA 1600	GTTA 285	Bulk[3]
74	7 (5)	+	+	+	+	-	+	
75	7	+	+	+	+	-	+	
77	4 (5)	-	-	-	-	+	-	
79	7	-	+	+	+	-	+	
81	8 (6,8)	+	+	+	+	-	+	
83	3	-	-	-	-	+	-	
84	8 (6,9)	+	+	+	+	-	+	V2
86	7 (6)	+	+	+	+	-	+	
87	1	-		-	-	+	-	A1
88	1 (2)	-		-	-	+	-	A1
89	7	+	+	+	+	-	+	
91	7	+	+	+	+	-	+	
92	3 (2,5)	+	+	-	-	+	-	A3
93	7	-	-	+	+	-	+	
94	2 (4)	-	-	-	-	+	-	
95	7 (6)	+	+	+	+	-	+	V2
96	7 (8)	+	+	+	+	-	+	V1
97	2 (1,3)	-		-	-	+	-	A1
98	3	-	-	-	-	+	-	
99	7	+	+	+	+	-	+	
100	4	-	-	-	-	+	-	
101	3 (4)	-	-	-	-	+	-	
102	1 (3)	-		-	-	+	-	A2
103	9 (8)	+		+	+	-	+	V2
105	1 (2)	-		-	-	+	-	A3
107	2 (3)	+		+	+	+	-	A2
109	7 (5)	+	+	+	+	-	+	
110	1 (2)	-		-	-	+	-	A1
1906	1	-	-	-	-	+	-	
1607	8 (7)	+	+	+	+	-	+	

[1] The most frequent rating (mode) is reported with other observed ratings in parentheses.
[2] Presence (+) or absence (-) of an AFLP marker.
[3] Indicates isolates used to generate bulked DNA samples (eg. V1 was bulk number one of highly virulent progeny).