Genotypic Analysis of *Rhynchosporium secalis* Pathotypes

by

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GENOTYPIC ANALYSIS OF *Rhynchosporium secalis* PATHOTYPES

BY

MARCY JOHNSON

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Abstract

Leaf scald of barley is caused by the imperfect fungus *Rhynchosporium secalis*. The incidence of this disease is increasing in western Canada yet little is understood about the genetics of the fungus or the mechanisms of resistance in the host. Inoculation of *R. secalis* onto differential barley cultivars shows great variation in virulence. The Canadian isolates used for infection of 24 barley cultivars appeared to cluster as two groups based on similarity of virulences. When RAPD primers were applied to the genomes of the same isolates and polymorphic fragments were compared, these two clusters disappeared. RAPD analysis also showed variability to be spread throughout the entire genome of *Rhynchosporium secalis*. Necrosis inducing peptide 1 is involved in a gene-for-gene relationship with *Rh3* of Atlas 46. If the peptide is present and active in the genome of an isolate, an incompatible reaction occurs. Sequence analysis of NIP 1 showed nucleotide changes which may affect the interaction with *Rh3* and therefore affect virulence/avirulence reactions.

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1. Introduction

Rhynchosporium secalis (Oud. Davis) is the causal agent of barley leaf scald. Scald disease has reduced the yield of barley crops in western Canada by up to 10% (Buchannon and Wallace, 1962). *Rhynchosporium secalis* causes lesions on barley leaves that are characterized by a grey centre with a dark brown margin. This organism can also infect other grass species such as *Agropyron repens* or *Secale cereale* (rye) and may be able to cross over from one species to another (Caldwell, 1937, Schein, 1958). Studies in Canada (Tekauz, 1991), Australia (Brown, 1990), and the southwestern United States (Jackson and Webster, 1976a) indicate that naturally occurring *R. secalis* populations have highly variable virulence patterns. A sexual state has not been identified for *R. secalis*, yet virulence can change rapidly within two generations (Jackson and Webster, 1976). Parasexuality and high mutation rates at virulence loci have been postulated as causes of heterogeneity (Newman and Owen, 1985, Goodwin et al, 1994). A clear understanding of the population structure of this pathogen would be useful for evaluating host resistance gene deployment strategies. Unfortunately, this type of understanding has been difficult to obtain using direct virulence screening on differential sets due to a loss of virulence in isolates and the lack of barley lines containing clearly defined, single resistance genes.

The high level of heterogeneity exhibited by this pathogen raises the question as to whether variation is limited to regions of the genome responsible for virulence only or is the entire genome variable. That is, could the main cause of

heterogeneity be uniformly high mutation rates or is it the result of certain regions being more prone to change such as transposon rich regions. Random amplified polymorphic DNA (RAPD) analysis uses arbitrary sequence 10 base primers to amplify random fragments of the genome (Williams et al, 1990). This technique allows for random sampling of loci for DNA sequence variation within the genome. RAPD analysis is also faster and more economical than other methods of DNA analysis (Williams et al, 1990). The polymorphisms identified with RAPD primers can be compiled and studied to determine relationships of organisms at the species level (Huff et al, 1994), or by geographic origin (Chen et al, 1993, Kolmer et al, 1995). In this study, RAPD analysis of the genomes of 31 *R. secalis* isolates resulted in a large data set of DNA polymorphisms. The data suggests that DNA sequence variation is spread throughout the entire genome and is not confined to sites responsible for (a)virulence.

The gene-for-gene hypothesis is the accepted theory for plant-pathogen interactions (Flor, 1942, 1955). This hypothesis explains specific host-pathogen interactions as involving two genes, one an avirulence gene in the pathogen, and the other a resistance gene in the host. If there is recognition between these gene products then an incompatible reaction occurs and infection is prevented. When there is no recognition event, a compatible reaction results wherein the pathogen is virulent and the host is susceptible (Flor, 1942). The gene-for-gene hypothesis can be interpreted differently at the molecular level for different systems. A common model involves an elicitor and receptor as the avirulence gene product and

resistance gene product respectively (Gabriel and Rolfe, 1990). The *Rhynchosporium secalis* / *Hordeum vulgare* system is an example of such a model (Rohe et al, 1995).

Because *R. secalis* grows subcuticularly on leaves and does not enter the host cell, the fungus must lyse the plant cell to release the nutrients required for its survival (Caldwell, 1937, Ayesu-Offfei and Clare, 1970, Lehnackers and Knogge, 1990). It has been shown that small molecular mass toxins are capable of causing cell death without physically penetrating the cell (Wevelsiep et al, 1991). Small molecular mass toxins were found in *R. secalis* culture filtrates and isolated as three separate necrosis inducing peptides (NIP) (Wevelsiep et al, 1991). Purified NIP 1 and 3 were found to non-specifically elicit stimulation of the K^+ stimulated, Mg^{+2} dependent plasmalemma H^+ATPase (Wevelsiep et al, 1993). The function of NIP 2 has not yet been elucidated. *R. secalis* isolates that carry an active form of the NIP 1 gene are avirulent on the barley cultivar Atlas 46. Atlas 46 was derived by combining the resistance from Turk (*Rh3)* with resistance from Atlas (*Rh2*). A specific interaction occurs between the NIP 1 gene of *R. secalis* isolates and the *Rh3* gene from Turk, as many isolates with NIP 1 that were avirulent on Atlas 46 were virulent on Atlas (Lehnackers and Knogge, 1990, Hahn et al, 1993). Following the gene-for-gene hypothesis, NIP 1 is the avirulence gene product from the pathogen *(R. secalis*) and *Rh3* is the resistance gene whose product is involved in recognition by the host (barley). Following the elicitor-receptor model, NIP 1 elicits a defense response when it is recognized by the *Rh3* gene product. When NIP 1

is not recognized (*Rh3* is not present in the host) it behaves as a virulence factor and stimulates the plasma membrane H^+-ATPase of the host cell to cause cell collapse (Lehnackers and Knogge, 1990). Pathotypes that are virulent on Atlas 46 have been isolated in western Canada. The DNA sequence of the NIP 1 gene [provided by Dr. Wolfgang Knogge (Max Planck Institut, Köln)] was used to characterize Canadian *R. secalis* isolates. DNA sequence analysis shows relevant differences between isolates that may be the cause of virulence or avirulence on barley cultivar Atlas 46.

2. Literature Review

2.1 *Rhynchosporium secalis*

2.1.1 Origin and nomenclature

Scald of rye was first reported in the Netherlands in June, 1897 by Oudemans and the pathogen responsible was described as *Marsonia secalis*. In October of the same year scald of barley was described by Frank in Germany who called the causal agent *Rhynchosporium graminicola*. In 1919, Davis realized that the two pathogenic fungi were the same and combined the nomenclature into the current name of *Rhynchosporium secalis* (Caldwell, 1937). *R. secalis* can infect a variety of grass species but the most common, and the most economically significant is barley (*Hordeum vulgare*). Reports are mixed about whether individual *R. secalis* races can infect more than one grass species. Caldwell (1937) found no evidence of crossover between host species yet Schein (1958) did.

2.1.2 Life cycle and general characteristics

Rhynchosporium secalis primarily infects the leaves of the host plant but can also infect stems and awns (Owen, 1973). Conidia are dispersed from plant to plant or leaf to leaf by water droplets and germination is self-inhibited until conidia contact appropriate water and nutrient conditions on the host leaf surface (Ayres and Owen, 1970, Owen, 1973). Within 24 hours, germination of the spore and direct hyphal penetration of the cuticle take place (Caldwell, 1937, Ayesu-Offei and Clare, 1970). Subcuticular mycelia growth follows the margins of adjacent

epidermal cells ultimately causing collapse of the outer epidermal wall and allowing access to mesophyll cells (Caldwell, 1937, Ayesu-Offei and Clare, 1970, Howlett and Cooke, 1987). Upon collapse of the mesophyll cells, the mycelia rapidly generate a fertile stroma using the nutrients provided by the broken cells. The stroma becomes the surface of the lesion as the cuticle is separated from the epidermis and lost (Caldwell, 1937, Ayesu-Offei and Clare, 1970). Conidia develop from conidial mycelia on the outside surface of the stroma and are splash dispersed thus repeating the cycle of infection (Caldwell, 1937, Ayesu-Offei and Clare, 1970, Howlett and Clare, 1987). Macroscopoic symptoms first appear as a bluish grey, watersoaked lesion that gradually develops into the characteristic grey spot with a dark brown margin upon mesophyll penetration by the mycelia. Sporulation only occurs on the side of the leaf that was initially infected and is most abundant in the centre of the lesion where cell collapse is the greatest (Caldwell, 1937, Ayesu-Offei and Clare, 1970). Conidia are beaked with a single septate (Owen, 1973). Germ tube growth and development are slow at low temperatures (2-4°C), and at temperatures higher than 30^0C conidial cells rupture. Maximum growth rates occur at 18-20^0 C (Caldwell, 1937). *R. secalis* overwinters on barley straw and can undergo five to eight cycles of sporulation in a single growing season if conditions are favourable with alternating wet and dry periods (Skoropad, 1966, Ayesu-Offei and Carter, 1971).

2.2 Population dynamics and variability

2.2.1 Differential screening

Rhynchosporium secalis is highly variable in its virulence on barley cultivars and in its morphology. In Canada, Tekauz (1991) characterized 111 isolates on 10 differentials to give 45 different pathotypes and Xue et al (1991) found 20 pathotypes on 5 differentials from 352 eastern Canadian isolates. Jackson and Webster (1976a) assigned 175 California isolates to 75 pathotypes on 14 differential cultivars. In Italy 100 isolates were characterized on 13 differentials to give 17 pathotypes (Ceoloni, 1980), and 203 Australian isolates grouped as 35 pathotypes on 21 host cultivars (Ali et al, 1976).

Although many attempts to determine races of the pathogen have been made, comparisons between studies are difficult because there is no widely accepted standard differential set of cultivars due to a lack of well defined resistance genes. Determination of the identity of resistance genes in barley cultivars is troublesome because of the variability of the pathogen. Environmental conditions play a role in pathogen variability, for instance, 10 isolates were classified into 8 pathotype groups when inoculated under fluctuating temperature conditions but when conditions were held constant, 122 isolates were differentiated into just 2 pathotypes (Owen, 1963).

Not all studies on virulence have exhibited high levels of heterogeneity. Of 319 Australian isolates from Victoria only 5 pathotypes were identified on a differential set of 15 host cultivars (Brown, 1985) and in the United Kingdom only 2 pathotypes were found in 122 isolates on 12 cultivars (Williams and Owen, 1973).

2.2.2 Virulence and fitness for survival

With no selection pressure on the pathogen for virulence (no increase in the resistance of the host population), *Rhynchosporium secalis* retains virulence potential in excess of what is required (Hansen and Magnus, 1973, Jackson and Webster, 1976b) yet isolates with more virulence are probably less fit for survival (Jackson and Webster, 1976b). Xue and Hall (1991) found that isolates with more virulence tend to require longer incubation times for spore germination and have reduced sporulation on susceptible hosts compared to less virulent isolates. The most frequently found races of *R. secalis* are the less virulent ones (Xue and Hall, 1991), but the trend towards simpler (less virulent) races may be an artifact of using the same differential set to assess virulence. The differential set used for one experiment might be a good representation of the resistance at the time and therefore capable of detecting the corresponding virulence of the pathogen. After years of introducing new resistance into the host, the same differential set would detect only the virulence needed to overcome the resistance of the original differential plants. Any additional virulence would not be seen, with the result that evolution of the pathogen would appear to move towards simplicity (Andrivon and deVallavieille-Pope, 1995).

2.2.3 Reasons for variability

Goodwin et al (1993) presented two ideas concerning the evolution of variability for virulence in scald populations: 1) *Rhynchosporium secalis* may have evolved

on wild barley at a centre of diversity and recently spread throughout the world. However, as the spread of this pathogen passed through geographic or ecological bottlenecks, genetic diversity would decrease. 2) *Rhynchosporium secalis* has been present on native grass hosts everywhere and just recently moved to barley. High levels of heterogeneity are maintained if the fungus switched from the variety of native grasses to barley at several different times although geographically separated populations should be highly differentiated. The results of isozyme analysis show little differentiation and therefore do not support this theory (Goodwin et al, 1993).

The genetic basis for variability can only be postulated, as *Rhynchosporium secalis* has no identified sexual stage. Parasexuality (Newman and Owen, 1985), (heterokaryosis followed by nuclear fusion and mitotic recombination (Burdon et al, 1994)), is an explanation for rapid shifts in virulence that may equal the rate of change with sexual recombination. However, parasexual recombination has not been observed outside of the laboratory and even then, it is rare for heterokaryosis, nuclear fusion and haploidization to all take place (Goodwin et al, 1994). Mutation is an alternate source of variation (Goodwin et al, 1994). If (a)virulence genes are located in an unstable portion of the chromosome, mutation rates can reach high levels as evidenced by the *avr2YAMO* gene in the rice blast pathogen *Magnaporthe grisea* (Valent, 1995). The *avr2YAMO* gene is located near the telomere where transposon movement, chromatin rearrangements, and point mutations may occur more frequently than in more proximal regions (Valent, 1995).

A change in pathotype can come about in two disease cycles. Five pathotypes were cycled through host plants twice resulting in 14 new pathotypes in the second infection. Approximately 75% of these differed by their virulence on only one or two differentials. In general, the population shifted towards more simple pathogenic races which infected fewer of the differential cultivars (Jackson and Webster, 1976b).

Zhang et al (1987) found a correlation between pathotype or disease index and geographic location. Disease index is a quantitative measure of virulence that allows for variations within rating categories such as moderately susceptible or moderately resistant reactions. The disease indices of 12 regions around the world varied significantly from area to area, thereby indicating geographic differentiation of virulence.

2.3 Genetic markers

2.3.1 Isozymes

An isozyme is an enzyme that is catalytically and structurally similar to another enzyme within the same organism (Voet and Voet, 1990).

2.3.2 Relationship to virulence

Isozyme experiments have been performed on *R. secalis* to determine whether variability for virulence was related to variability in other regions of the genome. Allelic variation for phosphogluco-isomerase (PGI), phospho-glucomutase (PGM),

leucine aminopeptidase (LAP), and β-glucosidase (BGLU) as well as for colony colour were examined by McDonald et al (1989) who found that haplotypes become non-randomly distributed in a population. Haplotypes are defined by a pattern of five numbers determined by assignment of a 1 for the fast variant of the isozyme or for a black colony and a 2 for the slow variant or cream coloured colony. For example, an isolate that is fast for PGI, PGM, slow for LAP, BGLU and is black coloured has a haplotype of 11221. A specific haplotype appears to establish itself on a single host plant and then spread to neighbouring plants. This was postulated because subplots of test plots in California each tended to have a different haplotype as the predominant type present (McDonald et al, 1989). Alternatively, isozyme variability within a population was greater than between populations (McDonald et al , 1989). Although haplotypes were geographically separate, there was no relation between the haplotype and the virulence or pathotype of the isolate. Ribosomal DNA (rDNA) sequence variation was studied in addition to isozyme and colour variation as an indication of genetic variability in the scald genome (McDermott et al, 1989). 29 rDNA RFLP phenotypes were found representing differences in the number of subrepeats in the spacer region between adjacent rDNA genes, however, no correlations to specific virulence or pathotypes were found (McDermott et al, 1989). Similar results were reported in other isozyme studies with relation to virulence in the United Kingdom and Australia (Newman, 1985, Burdon et al, 1994 respectively).

2.3.3 Allelic variation

Goodwin et al (1993) found 21 putative alleles at eight isozyme loci with an average of 2.63 alleles per locus. This pattern of isozyme variation is suggestive of the general presence of multiple alleles at any given locus with no null alleles. High levels of variation are maintained within a geographic location but there are few differences among geographically separated *R. secalis* populations, that is, the same alleles were found throughout all populations. These same conclusions were reached with isozyme studies in the United Kingdom (Newman, 1985) and in Australia (Burdon et al, 1994).

2.3.4 RFLPs

A restriction fragment length polymorphism (RFLP) is the result of a sequence change at a restriction enzyme target site that results in a difference in the length of fragment produced upon cleavage with the concerned enzyme (Lewin, 1990).

RFLP markers have not yet been applied to the *Rhynchosporium secalis* genome but are a useful tool for genetic studies. RFLPs were identified in *Verticillium* species that could be used to differentiate *V. albo-atrum* from *V. dahliae* (Carder and Barbara, 1991). RFLPs identified U.S. strains of the bacterium *Xanthomonas campestris oryzae* as genetically unique from other geographic origins (Xu and Gonzalez, 1991). RFLPs are also commonly used in mapping as genetic markers such as the rust resistance gene marker in *Triticum tauschii* (Gill et al, 1991).

2.3.5 RAPDs

Randomly amplified polymorphic DNA (RAPD) analysis is a technique that uses arbitrary sequence 10 base primers in a polymerase chain reaction with the genomic DNA of interest. The primers amplify fragments of the genome and are capable of describing polymorphic loci if there is even a single nucleotide sequence difference between two genomes at the site of primer binding (Williams et al, 1990, Welsh and McClelland, 1990). RAPD fragments have been used in crop species as linked markers for traits of agronomic importance and genome maps (Penner, 1995). Chen et al (1993) compared RAPD analysis of *Puccinia striiformis* races to the virulence spectrum of the isolates and found that there was little correlation between the two, although, the RAPD data did show evidence of the evolutionary relationships of isolates. These relationships were derived by cluster analysis of RAPD fragments. RAPD analysis has also been used to differentiate between species and individuals within a species for *Magnaporthe poae* (Huff et al, 1994). In *Cladosporium fulvum*, RAPDs were used to differentiate between single copy and repetitive DNA (Arnau et al, 1994). A second round of amplification conducted on single amplicons excised from agarose gels resulted in a number of bands. When probed with the original RAPD primer, Southern blot analysis showed that all of the reamplified fragments contained binding sites, indicating that the original band was made up of repeats.

2.4 Necrosis inducing peptides

2.4.1 Gene-for-gene

The gene-for-gene hypothesis was first presented by H. H. Flor (1942) and concerned his work with the *Melampsora lini/Linum usitatissimum* system. It has since been adopted as the basic theory for all host/pathogen interactions. Originally stated as "the range of pathogenicity of a physiologic race of *Melampsora lini* is determined by pathogenic factors specific for each resistance factor possessed by the host" the hypothesis suggests that for a specific interaction between host and pathogen, a recognition event between the pathogen's avirulence gene product and the resistance gene product of the host takes place which triggers host defense mechanisms. If either the avirulence gene product or the resistance gene product is absent, a compatible or virulent reaction results. The specific gene-for gene interaction is superimposed on basic compatibility resulting in two levels of plant - pathogen contact (Knogge, 1991). Basic compatibility is the ability of a pathogen to infect a host species by avoiding the general defense responses of the plant (Knogge, 1991). The gene-for-gene system may have evolved from a symbiotic relationship between fungus and plant. For example, a symbiotic fungus may have acquired a virulence factor that was detrimental to the host thereby exerting pressure on the plant to develop a specific gene for resistance. This interaction continues until both the host and pathogen have developed genes for specific recognition (Heath, 1987). A common model of the gene-for-gene hypothesis is the elicitor-receptor model wherein the avirulence gene product is recognized by a

receptor encoded by the host resistance gene and elicits a defense response (Gabriel and Rolfe, 1990). Other models include specific interactions determined by the resistance gene and the virulence gene rather than the avirulence gene (Vanderplank, 1982), and specificity controlled by the susceptible and virulence genes instead of the resistance and avirulence genes (Wheeler, 1975). Alternative models arose out of a need to apply the hypothesis to a broad spectrum of host-pathogen interactions. The *Rhynchosporium secalis-Hordeum vulgare* interaction is thought to be of the elicitor-receptor type (Knogge, 1991).

2.4.2 Necrosis inducing peptides and toxins

The first indication of toxin involvement in scald disease came in 1971 when Ayesu-Offei and Carter found that sterile culture filtrates could cause disease symptoms on barley and other host grasses. Leaves inoculated with spores of *R. secalis* had increased respiration rates and higher levels of nutrients in the intercellular spaces of infected leaves than non-infected leaves (Ayesu-Offei and Carter, 1971, Jones and Ayres, 1972). These results imply that nutrients are being removed from the cell without any direct penetration of the host cell by the fungus. The first toxic peptides identified were called rhynchosporosides and were low molecular weight 1,2-propanediol glucosides (Auriol et al, 1978). These rhynchosporosides could cause necrosis of leaf tips and margins but were not linked to specific host-pathogen interactions (Wevelsiep et al, 1991).

In 1990, Lehnackers and Knogge found that *R. secalis* isolate US238 could infect

the barley cultivar Atlas but not the near isogenic cultivar Atlas 46 which has an additional resistance gene, *Rh3,* derived from the cultivar Turk (Riddle and Briggs, 1950). Upon closer examination of infection of both cultivars with US238, they found that spore germination and germ tube growth were inhibited in the incompatible reaction on Atlas 46. The presence of the *Rh3* gene did not correlate with inhibited spore germination but the gene product may function to limit hyphal growth (Lehnackers and Knogge, 1990).

Further studies on isolate US238 showed that 3 necrosis inducing peptides (NIP) were produced during the infection process (NIP 1, 2 and 3) (Wevelsiep et al, 1991). These peptides are appropriate candidates for causing disease symptoms because they are small enough to easily diffuse across cell membranes. NIP 3 is a 9.2 kDa glycoprotein. Upon removal of the glycomoiety, NIP 3 is 5.4 kDa and retains its function. The glycoportion is high in mannose or complex carbohydrate chains and is linked by an N-glycosidic bond to the protein (Wevelsiep et al, 1991). NIP 2 is 6.8 kDa, and NIP 1 is 3.8 kDa. NIP 1 exhibits an apparent molecular weight of 7.2 kDa in the absence of a reducing agent, indicating a high number of disulfide bridges. (amino acid sequence shows 10 cysteine residues in the first 52 amino acids of the N-terminal sequence) (Wevelsiep et al 1991). All three of these peptides cause necrosis non-specifically when applied to host leaves, but toxin application alone does not necessarily show specificity (Knogge, 1991, Wevelsiep et al, 1991). There could be differential production or suppression of the toxin in the pathogen, or the host may tolerate or degrade the toxin upon infection by the entire

pathogenic organism (Wevelsiep et al, 1991).

2.4.3 Function of necrosis inducing peptides

NIP 1 and NIP 3 both stimulate the plasmalemma H^+ ATPase of the host cell when mixed with plasmalemma membrane vesicles. Partially purified ATPase was not stimulated by either NIP indicating that they do not interact directly with the H^+ ATPase (Wevelsiep et al, 1993). The function of NIP 2 is unknown. NIP 3 and NIP 2 are found in all isolates analyzed and are not specific elicitors of the host defense response. NIP 1 appears to impart some host specificity as it is present in isolates which are avirulent on host cultivars carrying the *Rh3* gene and absent or non-functional in isolates that are virulent on cultivars with *Rh3*. Atlas 46 received its version of the *Rh3* locus from Turk (Riddle and Briggs, 1950). Both Atlas 46 and Turk are resistant to races carrying the NIP 1 gene. Other cultivars that are thought to have the *Rh3* gene for resistance do not give the same differential responses to NIP 1 therefore the resistance gene designation for these cultivars is incorrect or they contain an alternate allele at the *Rh3* locus (Hahn et al, 1993).

2.4.4 ATPase stimulation

It appears that the stimulatory effect of NIP 1 on ATPase is the result of the stimulation of the plasmalemma Mg^{++} dependent, K^+ stimulated H^+ ATPase by NIP 1 in the same manner as fusicoccin from *Fusicoccum amygdali* (Meyer et al, 1989). That is, there is no direct interaction with ATPase and elicitation must occur through

an intermediary (Wevelsiep et al, 1993). The elicitor effect of NIP 1 can be separated from the stimulatory effect. NIP 3 is also able to stimulate the plasmalemma ATPase but it does not elicit a cultivar specific host response (Wevelsiep et al, 1993). ATPase controls the internal and external pH and therefore has indirect effects on all cell activities that are dependent on the proton gradient across the plasma membrane including nutrient uptake (Serrano, 1989). ATPase can be regulated by kinases or by ion concentrations and malfunction of the plasmalemma ATPase can cause cell collapse due to loss of osmotic pressure.

2.4.5 NIP 1 as a host specific elicitor

In the incompatible host pathogen interaction, NIP 1 causes rapid accumulation of host pathogenesis related proteins and peroxidases which presumably results in prevention of infection. In particular, high levels of mRNA for PRHv-1, which has homology to a thaumatin-like protein, are rapidly produced within 24 hours post inoculation (Rohe et al, 1995). PRHv-1 is most likely a basic defense response related protein but by triggering production of many copies of the mRNA, NIP 1 may be linking cultivar specific reaction to basic host-pathogen defense (Heath, 1981, Knogge, 1991). In the susceptible cultivar Atlas, PRHv-1 accumulates but at a much slower rate than in the resistant cultivar Atlas 46. This could indicate that the receptor for NIP 1 in Atlas coded for by *rh3* is much weaker than the one in Atlas 46 coded for by *Rh3* (Hahn et al, 1993).

Experiments carried out by Rohe et al (1995) confirm that NIP 1 is an avirulence

factor. When NIP 1 was co-inoculated with a virulent isolate onto Atlas 46 it exhibited avirulence, and when the *avrNIP* 1 gene was transformed into the genome of a virulent isolate it exhibited an avirulent reaction. Amino acid sequencing of NIP 1 showed that it is composed of 82 amino acids, the first 22 of which are a secretory signal peptide, leaving 60 amino acids as the mature protein. DNA sequencing indicates a 65 base pair intron before the three carboxy terminus amino acids (Rohe et al, 1995). Certain isolates are virulent on Atlas 46 and appear to contain a modified form of the NIP 1 gene (Rohe et al, 1995). Elicitor assays showed that in these isolates, there was no elicitor activity. It also appears that the most virulent isolates are those that carry an elicitor inactive form of the NIP 1 avirulence gene rather than those that completely lack NIP 1, thereby implying that the peptide retains its virulence properties even though it is no longer an elicitor of host defenses (Rohe et al, 1995).

2.5 Host resistance

There is evidence that resistance genes in the host may have a complementary effect. Australian isolates assigned to pathotypes aust.1 and 10 were both virulent on Atlas and Turk, the parents of Atlas 46, yet were avirulent on Atlas 46 (Ali et al, 1976). There have been 13 genes identified for seedling resistance to scald disease to date (Graner and Tekauz, 1996). It appears that at least three of these genes (*Rh, Rh3* and *Rh4*) are clustered on the long arm of chromosome 3 although the structure of the cluster is unknown (Graner and Tekauz, 1996). Adult resistance

to *R. secalis* that is different from seedling resistance has been identified in some barley cultivars (Xue et al, 1995) and slow scalding targets plants at the adult stage only (Kari and Griffiths, 1993). In spite of this, seedling resistance remains a good indication of adult plant resistance and requires less time and space for experiments (Xue et al, 1995). Jackson and Webster (1976a,b) provided strong evidence that the genetic basis of scald resistance in cultivars commonly used in differential sets is far more complex than has been reported.

3. Materials and Methods

3.1 Scald Inoculation

3.1.1 Culture maintenance

Single spore *Rhynchosporium secalis* cultures were isolated as described by Tekauz (1991). Leaves collected from infected plants in the field were stored in paper envelopes for no longer than two months before isolation of the pathogen. 5 x 2 mm sections of infected leaf tissue were surface sterilized in 50% ethanol for 15 s, in 2% sodium hypochlorite for 30 s, then rinsed in sterile distilled water. Sterilized leaf sections were embedded in lima bean agar (Difco) on a petri plate and incubated at 20°C with 12 h light and 12 h dark / 24 h. Incubation continued for 7-10 days then single colonies were diluted in sterile water and streaked onto fresh LBA plates. After incubation, small discrete colonies grown from a single spore were transferred to test tubes containing potato sucrose peptone agar slants (bacto peptone 10 g/l, agar 10 g/l) and allowed to establish growth (3-4 weeks) before being flooded with mineral oil and stored at 4°C in the dark. To restart growth, a portion of the agar containing mycelia stored under oil was transferred to Fries medium (ammonium tartrate 27 mM, NH_4NO_3 12.5 mM, $MgSO_4 \cdot 7H_2O$ 0.2 mM, KH_2PO_4 9.6 mM, K_2HPO_4 14.9 mM, sucrose 87.6 mM, yeast extract (Difco) 1g/l and 0.2 % trace elements) with a sterile transfer loop and incubated at 18-20°C with 12 h light and12 h dark / 24 h.

3.1.2 Inoculum preparation

R. secalis cultures in Fries medium, grown for 2 to 3 weeks, were transferred to potato sucrose peptone agar slants to inoculate the surface of the slant and incubated. After one week, slants were flooded with 8 ml of sterile distilled water, scraped with a sterile transfer loop then spore suspensions were poured into 40 ml potato sucrose solution (sucrose 50 g/l in 1:1 potato water:sterile water) and grown for an additional seven days. All incubations were at 18-20°C with 12 h illumination /12 h dark.

To prepare spores for inoculation, one to five bottles per isolate of incubated suspension culture were covered with parafilm and shaken to dislodge spores, blended in a Waring blender for a minimum of 45 s and filtered through two layers of cheesecloth into a non-sterile flask. The concentration of these spore suspensions was measured on a Klett spectrophotometer using sterile water as a blank. If a minimum spore concentration of 600,000 was not reached, the culture bottles were re-shaken with sterile water or another broth culture of the same isolate was used and the procedure repeated. Spore concentrations between 600,000 and 1,000,000 spores per ml were used. A drop of the spore suspension was examined under a light microscope to ensure the presence of spores. If there were more mycelia present than spores, the suspensions were re-blended, filtered, and the Klett reading repeated. One drop of Tween 20 was added per 50 ml of inoculum as a sticker agent. Viability of spores was determined by plating a drop of inoculum onto PSPA medium plates and assaying for growth one week later.

3.1.3 Inoculation and Rating

Barley differentials were planted four to a pot with 5 seeds per differential in a 4:1:1 (soil : sand : turface) soil mixture and fertilized with approximately one half a teaspoon of 34:0:0 (N:P:K). Plants were fertilized again with 20:20:20 (N:P:K) the day before inoculation, in the final inoculation experiment only. After growing for 2-3 weeks, the seedlings were inoculated with freshly prepared spore suspension. Inoculum was applied at a rate of 15 ml per pot with an atomizer fitted to an air pump. The atomizer was rinsed with 70% ethanol and then sterile distilled water before applying inoculum and after each isolate was applied.

Prior to returning the plants following inoculation, the growth cabinet was misted with water to produce a highly humid environment and the lights were turned off. Efforts were made to ensure that the leaves of plants inoculated with different isolates of the pathogen did not come into contact with each other. Incubation continued in the dark for 24 h at 18-20°C, then lights were turned on for two hours, for the health of the plants, before resuming darkened conditions for the remainder of the first 48 h. During the 48 hours of incubation, the cabinet was misted 3-5 times. The light schedule following the dark period was 16 h light /8 h dark.

Rating of disease severity was performed two weeks post inoculation with supplementary rating in some cases at three and four weeks post inoculation. Ratings were divided into four infection types, resistant (R), moderately resistant (MR), moderately susceptible (MS) and susceptible (S) according to the system used by Tekauz (1991) (Table 1).

Table 1 Four categories for rating of scald disease severity

symbol	rating	symptoms
R	resistant	no disease symptoms
MR	moderately resistant	small lesions confined to the leaf tips and margins
MS	moderately susceptible	larger lesions and moving toward the centre of the leaf
S	susceptible	large coalescing lesions or total collapse of the leaf

3.2 Genetic Analysis

3.2.1 DNA extraction

DNA was extracted using a modified form of the McDermott et al (1989) method. *Rhynchosporium secalis* cultures were grown for 2-4 weeks in Fries medium before harvesting mycelia for DNA extraction. Mycelia were harvested by shaking and filtering the broth culture through Miracloth in a Buchner funnel under vacuum. 1-1.5 g of filtered mycelia were placed into a pre-chilled mortar with liquid N_2 and ground to a fine powder with a pestle and then suspended in 3 ml of extraction buffer (150 mM EDTA, 50 mM Tris pH8.0, 1% sarcosyl, and 300 mg/l proteinase K). These suspensions were transferred to 15 ml Falcon tubes, vortexed for 45 s, and then centrifuged at 2100 rpm for 10 min. The supernatant was extracted twice with an equal volume of chloroform : phenol : isoamyl alcohol (25:24:1). The McDermott et al. (1989) protocol includes extraction of the aqueous phase with ether three times. This ether step was included in one extraction procedure but did not greatly increase DNA yield and was therefore omitted in further extractions. The aqueous phase was precipitated by the addition of two volumes of 95% ethanol and 0.25 M final concentration of sodium acetate and incubation at -20°C for a minimum of 20 min (usually an hour or more). After centrifugation for 10 min at 2100 rpm the pellet was washed with 70% ethanol and re-centrifuged. The dried pellet was resuspended in 200 - 500 µl TE buffer, depending on the size of the pellet. Some extractions were treated with 10 units of RNAse (Boehringer Mannheim) at 37°C for one hour while the RNA in other extractions was allowed to degrade over time.

There was no difference detected in RAPD results with or without the presence of RNA. Concentrated stocks of suspended DNA were stored at 4°C. The concentration of extracted DNA was determined by A_{260} readings on a Varian DMS 100 spectrophotometer.

3.2.2 PCR conditions

Polymerase chain reactions for amplification of DNA were performed in a total volume of 25 μl. The reaction mixture consisted of 20 pM primer, 25 ng genomic DNA, 0.8 mM dNTPs (0.2 mM each of ATP, GTP, TTP, and CTP) (Promega), 2.0 mM $MgCl_2$ (Perkin Elmer), 1x Taq activity buffer (50 mM Tris/HCl pH9.0, 20 mM NaCl, 1% (v/v) Triton X-100) (Perkin Elmer), and 1 unit of Taq polymerase (Perkin Elmer). Barnstead Thermolyne Temptronic (model DB66925) thermocyclers were used for all amplification experiments.

3.2.3 RAPD-PCR

Thermocycler programs started with 5 min. at 95°C followed by 35 cycles of: 95°C for 5 s, 36°C for 30 s, and 72°C for 60 s. The final step was a 72°C dwell for 10 min before holding reactions at 5°C until amplification products were either moved to storage at -20°C or loaded onto an agarose gel. Primers used for RAPD experiments were 10 base oligomers of arbitrary sequence from the University of British Columbia (UBC) (Nucleic acid protein unit, 6174 University Blvd, Vancouver,

B.C. V6T1Z3) or from Operon technologies inc. (OP) (1000 Atlantic Ave. Alameda, Calif. 94501).

3.2.4 Gel electrophoresis

PCR products from the RAPD experiments were visualized on agarose gels. 1.6% agarose gels were run horizontally in 1x TAE (Tris 2 M, Acetic acid 0.0571% (v/v), EDTA 0.05 M) running buffer on either a BRL Horizon 20-25 or model H4 electrophoresis unit for 280 volt hours. 3 μl of loading buffer (sucrose 1.17 M, EDTA 0.12 M, bromophenol blue) were added to each PCR reaction (25 μl), 23 μl of this mixture were loaded per well. 3-4 μl of lambda DNA (1 μg/μl) digested with EcoR1/HindIII were used as a size marker. Ethidium bromide was added to the gel before casting at a concentration of 0.24 μg per ml of agarose. Ethidium bromide stained DNA fragments were illuminated on a Fisher 312 nm variable intensity, ultra violet transilluminator. DNA bands were photographed with either Polaroid type 57 or 667 film at exposure times of 1-1.5 s.

3.2.5 Data analysis

NTSYS-pc version 1.6 (Rohlf, 1985) was used to analyze the RAPD data. Polymorphic bands were scored as a 1 if present, or as 0 if absent. Similarity was assessed using qualitative genetic analysis and clusters were constructed using the UPGMA (unweighted pair group arithmetic mean) method of SAHN clustering. When ties were found they were not significantly different from the initial cluster and

therefore the original cluster formed was used in all cases. Dendrograms were produced from the clustered matrix to graphically illustrate relationships.

3.3 NIP 1

3.3.1 PCR conditions

To amplify the NIP 1 gene, the same time and temperature profile was used as in the RAPD experiments except for the annealing temperature. The optimum annealing temperature for specificity and yield was 52°C, however, annealing temperatures of up to 60°C would show amplification. Primers were initially provided by Dr. Wolfgang Knogge of the Max Planck Institute in Koln, Germany along with the NIP 1 sequence. When primer stocks were depleted, new oligomers were synthesized on an ABI PCRmate 391. Primer 3 (forward): 5'-CCTCCAAC TGAACCAAAAC -3'. Primer 4 (reverse): 5'-GCGCGATGCACAGTTCTTC-3'. Amplification of the barley genome with the NIP 1 primers (45°C annealing temperature) was also attempted.

3.3.2 Gel electrophoresis

1.6% agarose gels were used to visualize the NIP 1 amplification products in the same manner as for the RAPD PCR products. A mini agarose gel apparatus (BioRad wide mini sub cell) was employed for smaller numbers of reactions at 150 volt hours.

3.3.3 DNA fragment isolation

DNA fragments were extracted from agarose gels by the freeze and squeeze method. DNA fragments were visualized by placing the ethidium bromide stained gel on the U.V. light box and illuminating with a hand held (Camag TL-900/u) U.V. light with a wavelength of 350 nm. Bands of appropriate size were cut with an ethanol sterilized razor blade, placed into 1.5 ml Eppendorf tubes with sterile forceps, and frozen at -20°C overnight. Agarose cubes were then squeezed by applying pressure with a thumb and the expelled liquid collected. The supernatant was extracted with an equal volume of chloroform : phenol (1:1), microfuged, and the aqueous phase drawn off and precipitated with 0.1 volume sodium acetate (3M) and 2x volume of 95% ethanol. Following incubation at -20°C for 1 hour, pellets were washed in 70% ethanol, then dried and resuspended in 10-20 μl of TE buffer, depending on pellet size.

To isolate the NIP 1 gene, products of the initial amplification of NIP 1 were separated on agarose gels. A 200 μl pipette tip was inserted into the band of interest then swirled directly into a fresh PCR mixture as substrate for a second round of amplification. This pipette poke method was also used to re-amplify a fragment that could not be visualized after the first amplification.

3.3.4 DNA sequencing

Two attempts were made to cycle sequence the NIP 1 gene by the dideoxynucleotide chain termination method using Promega's kit for cycle

sequencing with the thermocycler program set at 94°C for 5 min. then 55 cycles of: 30 s at 95°C, 30 s at 55°C and 1 min. at 72°C. At the end of the program 3 μl of stop solution was added either before storage at -20°C or prior to heating at 80°C for 10 min. and loading onto the sequencing gel. The sequencing gel was composed of 5% acrylamide, 7.0 M urea, and 1x TBE buffer and was poured into a BioRad sequi-gen sequencing gel apparatus. The gel was run at 1800 V for 3.5 hours. To read the gel, bands were developed with a silver staining kit (Promega). The majority of sequencing was performed however, by the Plant Biotechnology Institute (NRC, Saskatoon) on lyophilized substrate.

3.3.5 Sequence analysis

To analyze sequence information, the computer program, "ALIGN" was used to compare the nucleotide sequence of necrosis inducing peptide 1 of different isolates and of the same isolate in different sequencing reactions. Sequence information was also screened on the Blast server for the purpose of finding identity with other organisms.

3.4 NIP 2

3.4.1 PCR conditions

Using the amino acid sequence of the NIP 2 peptide provided by Dr. Knogge (Max Planck Institut für Züchtungsforschung, Department of Biochemistry, D-50829 Köln Germany) we derived and synthesized a pair of degenerate primers for PCR

amplification NIP2A (forward): 5'-ATIGGIGAIGTIGAITGGGC-3' and NIP 2B (reverse): 5'-ACIACITCIACIACIGCA/GTT-3'. The degenerate primers had an optimum annealing temperature of 52°C with the same thermocycler profile as for the RAPD experiments. There was some difficulty amplifying the specific gene product without background amplification, therefore both the "hot start" method and "touchdown PCR" were used. The hot start method involves addition of Taq polymerase to the reaction mixture once the tubes are in the thermocycler block and the temperature is above 80°C. Hot start PCR helped to prevent non-specific amplification. Touchdown PCR entails a more complex temperature profile, the annealing temperature starts 10°C above the Tm of the primers and drops one degree each cycle until the desired annealing temperature is reached then cycles 25 times at that annealing temperature. This method also ensured more specific amplification products. It was determined that the NIP 2 degenerate primers worked best when mixed in a 1:4 ratio, that is, 8 pM of the forward (NIP 2A) and 32 pM of the reverse (NIP 2B) primer with the regular PCR program and an annealing temperature of 52°C.

Once the nucleotide sequence of a portion of the NIP 2 gene was determined, specific primers were designed: NIP 2E (forward): 5'-GGAGGTGGAGTGGGCAAT TCA-3' and NIP 2F (reverse): 5'-ACGTCGACGACGGCGTTTG-3'. These primers were also used with the 52°C annealing temperature and same cycling program as for the RAPD experiments. Hot start PCR and touchdown were also used with the NIP 2 specific primers.

3.4.2 Gel electrophoresis

Agarose gels were not sufficient to resolve the NIP 2 fragment from other background products, therefore acrylamide gel electrophoresis was used. Gels of 5% acrylamide provided the best resolution. All acrylamide gels were run on a BioRad Protean II xi Cell electrophoresis unit for approximately 600 volt hours with either 1x TAE or 1x TBE running buffer. Gels were stained in ethidium bromide for 30-45 min. and rinsed in ddH_2O before being photographed. Photographs were taken the same way as with agarose gels.

3.4.3 DNA fragment isolation

The NIP 2 gene was isolated from agarose by the freeze and squeeze method as above. For purification of the NIP 2 fragment from acrylamide, the appropriate band was cut from the gel with an ethanol sterilized razor blade and removed to a round bottom falcon tube with sterile forceps. The tube was then frozen at -20°C for storage. DNA was extracted from the acrylamide by cutting the fragments into smaller pieces to increase surface area followed by suspension in 500 μl of TE buffer (pH7.5). Centrifugation was used to separate the DNA from the acrylamide and tubes were incubated at 37°C overnight. The next morning, after centrifugation, the supernatant was used directly as substrate in PCR reactions or was further purified by a choloroform:phenol (1:1) extraction.

3.4.4 DNA sequencing

Purified NIP 2 gene fragments were sent to Plant Biotechnology Institute (NRC, Saskatoon) for sequencing reactions.

3.4.5 Sequence analysis

NIP 2 sequences obtained with the degenerate primers were compared to one another using the ALIGN program and identification of the sequence with other known DNA sequences was checked on the Blast server.

3.5 NIP 3

3.5.1 PCR conditions

Primers for the NIP 3 gene were derived from the amino acid sequence of the peptide determined by Dr. Knogge and a consensus of other fungal codon usage. These primers were: NIP 3C (forward): 5'-GATGTTTATACCATTGG-3' and NIP 3D (reverse): 5'-TCTTTTTTATCTTGATCAAA-3' with an optimum annealing temperature of 48°C. The same basic thermocycler profile as used for the RAPD amplifications was used for NIP 3 gene amplification. Both hot start and touchdown PCR methods were applied to NIP 3 amplification as well.

3.5.2 Gel electrophoresis

NIP 3 gene amplification results were visualized on 1.6% agarose gels as above or on 5% acrylamide gels as above.

3.5.3 DNA fragment isolation

The potential NIP 3 gene was excised from an agarose gel and frozen in preparation for freeze and squeeze extraction of the DNA. Freeze and squeeze was performed as above. Sequence analysis of NIP 3 was not done as a purified single fragment could not be obtained.

4. Results

4.1 Scald inoculation

Rhynchosporium secalis isolates were chosen for this study on the basis of heterogeneity in virulence based on previous characterizations (Tekauz, 1991) and unpublished results. Scald isolates were inoculated onto a differential set of cultivars as described in Table 2. Isolates WRS1824, WRS1859 and WRS1860 had only recently been collected. The *R. secalis* isolates applied to the cultivars comprising the differential set came from across Canada and had been collected as early as 1968 (Table 3).

Two to four weeks post inoculation, the *R. secalis* infected barley plants were scored for disease severity. Our rating system consisted of four levels of disease: R for resistance (no scald symptoms), MR for moderately resistant (small lesions confined to the leaf tip and margin), MS for moderately susceptible (larger lesions coalescing towards centre of leaf), and S for susceptible (total collapse of leaf tissue) (Table 1). Ratings of scald symptoms were repeated at least two times for each trial with an interval of 2 days to 1 week between each scoring. Ratings of R and MR were considered resistant and ratings of S and MS considered susceptible for comparative purposes. Results that were difficult to interpret were also scored by Dr. Andy Tekauz. If a cultivar exhibited susceptibility to an isolate in only one trial, it was still classified as susceptible (Table 4). We attempted to improve the screening procedure by using a detached leaf inoculation method but the cut barley leaves could not be maintained in culture for the minimum two weeks required for

Table 2 *Rhynchosporium secalis* isolates, inoculation dates and differential cultivars used.

Isolate number	Date inoculated	No. of differentials
92-BSMV#98	January 20, 1994	24
	December 19, 1994	8
WRS837	January 20, 1994	24
	March 1, 1995	8
WRS1380	January 20, 1994	24
	March 28, 1994	24
WRS1389	January 20, 1994	24
	August 17, 1994	24
	December 19, 1994	8
WRS1391	January 20, 1994	24
	March 1, 1995`	8
WRS1402	January 20, 1994	24
	March 1, 1995	8
WRS1426	March 28, 1994	24
	August 17, 1994	24
	December 19, 1994	8
WRS1474	January 20, 1994	24
	December 19, 1994	8
WRS1483	January 20, 1994	24
	March 28, 1994	24
	August 17, 1994	24
	December 19, 1994	8
WRS1488	January 20, 1994	24
	October 19, 1994	24
WRS1493	October 19, 1994	24
	March 1, 1995	8
WRS1780	January 20, 1994	24
	October 19, 1994	24
WRS1784	January 20, 1994	24
	March 28, 1994	24
	August 17, 1994	24
	December 19, 1994	8
WRS1824	March 1, 1995	8
WRS1859	March 1, 1995	8
WRS1860	March 1, 1995	8

Table 3 Origin of *Rhynchosporium secalis* isolates and collection information.

Isolate number	Location	Who collected	Date
92-BSMV#98	Glenlea, Manitoba	Eric Mueller	July 23, 1992
WRS837	Central Alberta	Dr. W. Skoropad	November 28, 1968
WRS1380	University of Guelph, Ontario	Dr. E. Reinbergs	July 13, 1978
WRS1389	Arnell Station, Ontario	Dr. E. Reinbergs	June 13, 1979
WRS1391	Elora, Ontario	Dr. E. Reinbergs	July 12, 1982
WRS1402	Ponoka, Alberta	Dr. S. Haber	July 11, 1985
WRS1426	Clyde, Alberta	Dr. P. Thomas	July 30, 1980
WRS1474	Beaverlodge, Alberta	Dr. A. Tekauz	July 26, 1985
WRS1483	White Gull, Alberta	Dr. A. Tekauz	July 31, 1985
WRS1488	Morwayne, Alberta	Dr. A. Tekauz	August 3, 1985
WRS1493	Tisdale, Saskatchewan	Dr. A. Tekauz	August 5, 1985
WRS1780	Sylvan Lake, Alberta		Summer, 1991
WRS1784	Manitoba	Eric Mueller	August 6, 1992
WRS1824	Pilot Mound, Manitoba		August, 1992
WRS1859	Souris, Manitoba		July 18, 1994
WRS1860	Olds, Alberta	Dr. P. Burnett	July 28, 1994
WRS1862*	Brandon, Manitoba	Dr. A. Tekauz	August 11, 1994
WRS1864*	Alberta Agriculture	Dr. P. Burnett	September15, 1994
MT 93*	Montana	Dr. K. Everts	1993
FF 94*	Montana	Dr. K. Everts	1994

* these isolates were included in RAPD experiments but not in virulence assessment.

Table 4 Matrix of combined virulence screening results of *Rhynchosporium secalis* isolates on 24 and 8 differential cultivars.

	1391	1389	1474	1780	1784	1488	1380	1426	1483	1402	1493	837	1824	1859	1860
1	-*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	+**	+	-	-	+	+	+	+	+	+	+	-	-		-
3	-	+	+	+	+	+	-	+	+	+	+	+	+	+	-
4	-	+	+	+	-	+	-	-	-	+	+	+	-	+	-
5	+	+	-	+	+	+	+	+	+	-	-	+	-		-
6	-	-	+	+	-	+	+	-	-	+	+	-	+	+	-
7	-	-	+	-	-	+	+	-	-	+	-	+	-		-
8	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-
9	-	+	+	+	-	+	-	-	+	-	+	+	-	+	-
10	-	+	+	+	-	+	-	+	-	+	-	+	-	+	-
11	-	+	-	+	-	+	-	+	-	+	-	-	-		-
12	-	+	+	+	+	+	-	-	-	-	-	-	+	-	+
13	-	-	-	-	-	+	-	-	-	-	+	+			
14	-	+	+	-	-	-	-	-	-	+	-	+	+		+
15	-	-	-	-	-	-	-	-	-	-	-	-			
16	+	-	-	-	+	-	-	+	-	-	-	+			
17	+	-	+	+	+	+	+	-	-	-	+	+			
18	+	+	+	+	+	+	+	-	+	-	+	+	+		+
19	-	-	-	-	+	-	+	-	-	-	+	-	-		+
20	-	-	-	+	+	-	+	+	+	-	+	+			
21	-	-	-	-	-	-	-	-	-	-	-	-			
22	-	-	-	-	-	-	-	-	-	-	-	-			
23	-	-	-	-	+	+	+	+	+	+	+	-			
24	-	-	-	-	-	-	-	-	-	-	+	-			

* - is susceptible, ** + is resistant, 1-24 are the numbers assigned to the cultivars for octal nomenclature as described in table 5.

the development of scald symptoms.

The results of the differential screenings with scald isolates were coded (1 for resistance and 0 for susceptibility) and a similarity matrix was constructed. Cluster analysis of the matrix produced a dendrogram that illustrates the degree of similarity among the isolates based on virulence (Figure 1). Two clusters emerged that were about 56% similar indicating the possibility of two different origins, migration events or a branch point in the evolution of virulence. The resistance most often found in barley cultivars in Canada seems to be *Rh* and *Rh2* (Penner et al, 1996). The two clusters of isolates that emerged are not based on geographic origin or date of collection but may be related to the resistance genes in the cultivars which they infect.

The differential cultivars that were inoculated were chosen from a suggested list (Goodwin et al, 1990). This list was compiled to standardize naming of *Rhynchosporium secalis* pathotypes (Table 5). The cultivars included in this list have been widely used to characterize virulence patterns and among them carry all the resistance genes identified to date. The differential set is intended to standardize naming of isolates by assigning octal names. Each binary number of the octal name is determined by the reactions of three cultivars, with similar or related resistance, to each isolate (resistance is assigned a 0 and susceptibility a 1). There are eight groups of three cultivars for a total of 24 cultivars. This system allows for missing data, smaller differential sets or addition of extra cultivars. The octal nomenclature for the Canadian isolates (Table 6) clearly show that there are

Figure 1. Dendrogram of *Rhynchosporium secalis* isolates based on the similarity of virulence on 24 barley differential cultivars

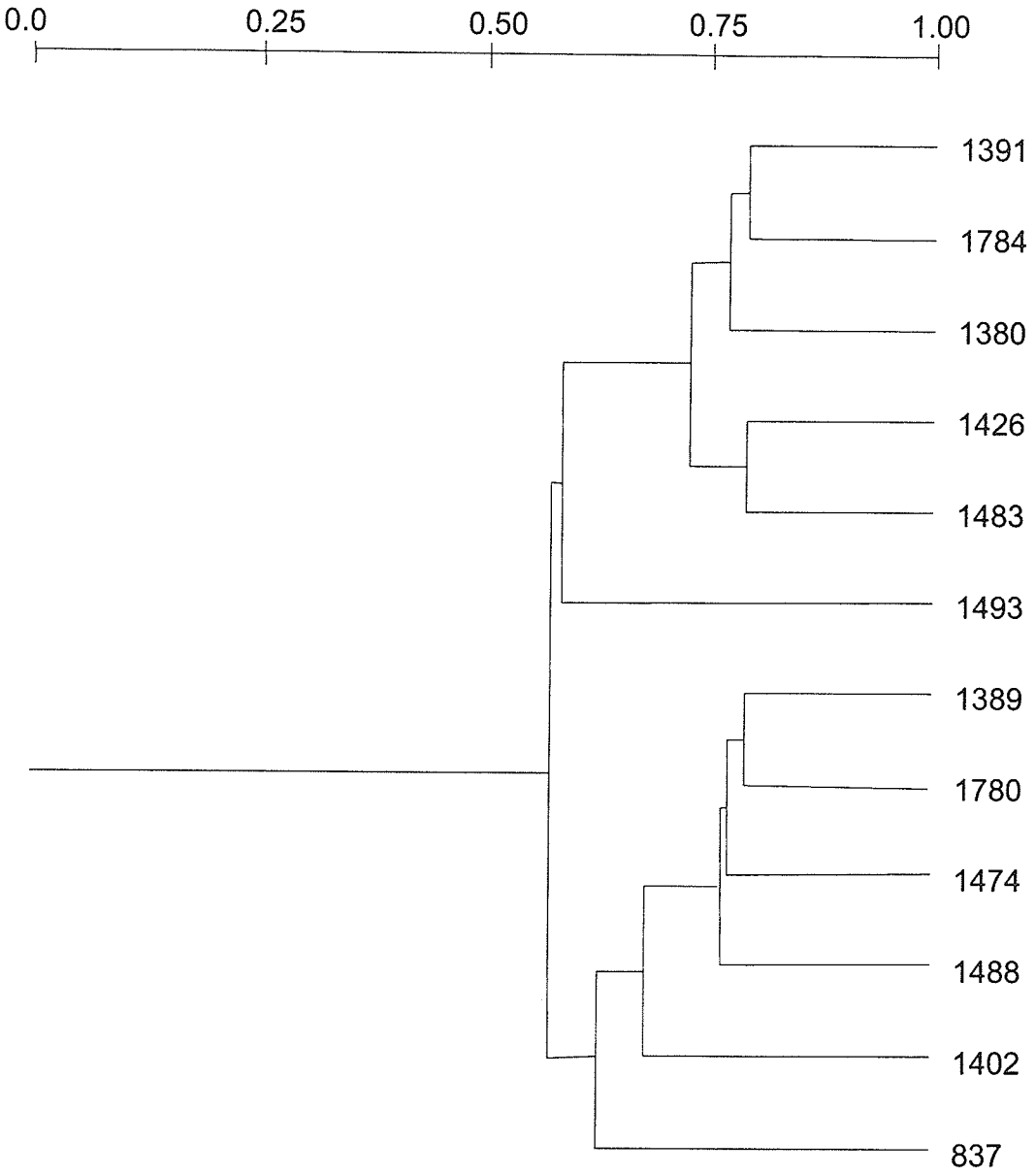

0.0
0.25
0.50
0.75
1.00
1391
1784
1380
1426
1483
1493
1389
1780
1474
1488
1402
837

Table 5 Assigned numbers for barley cultivars and lines used in octal nomenclature inoculations.

Number	Cultivar /lines
1	Harrington*
2	La Mesita
3	Atlas 46
4	WW x G
5	Brier
6	Hudson
7	Modoc
8	Trebi
9	Turk
10	Atlas
11	Steudelli
12	Osiris
13	Kitchin
14	CI 2376
15	CI 5831
16	CI 4364
17	CI 8618
18	CI 3515
19	Nigrinudum
20	West China
21	CI 3058
22	Gospeck
23	Sakigake
24	Sultan

* universal suscept

Table 6 Octal nomenclature for *Rhynchosporium secalis* isolates.

Isolate	Octal name
WRS837	75046043
WRS1380	54177615
WRS1389	77350341
WRS1391	77077755
WRS1402	57754621
WRS1426	55674751
WRS1474	77155223
WRS1483	55377351
WRS1488	57160201
WRS1493	14167121
WRS1780	75170303
WRS1784	54073751
WRS1824	1003733
WRS1859	4001
WRS1860	0003777

Underlined numbers indicate a missing data point within a triplet (considered resistant) and less than eight numbers indicates a smaller set of differentials.

no two isolates with the same virulence on the set of 24 cultivars. The binary numbers of the octal name are also helpful in determining the overall virulence of an isolate. The closer the binary number is to seven the more virulent the isolate is for that portion of the octal. The cultivars of the differential set are ordered so that the most resistance is in the third position (leftmost or closest to the bottom) of each triplet of cultivars (Table 5) therefore giving a higher binary number and an overall higher octal number.

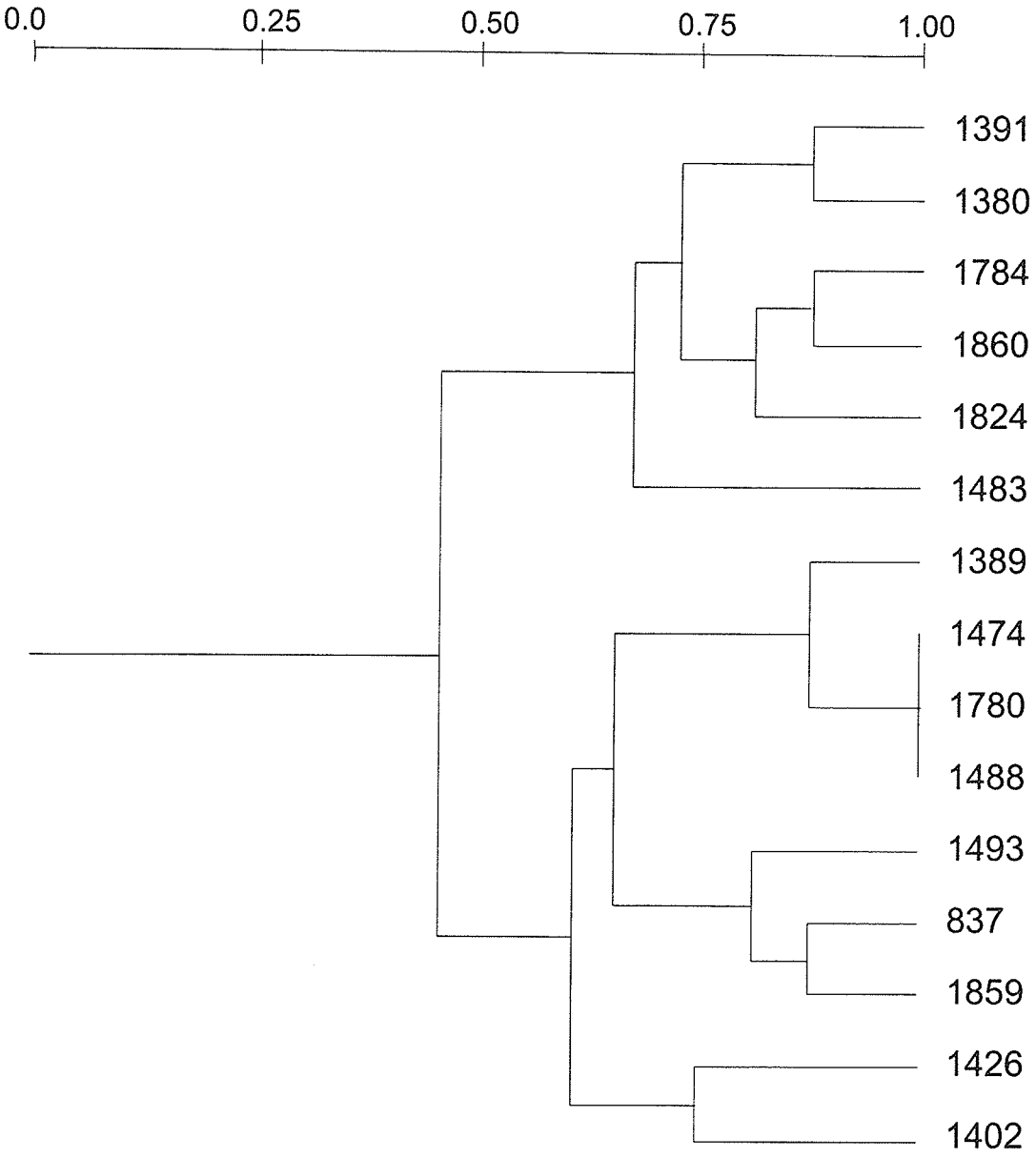
Using octal nomenclature as a comparative measure, the Canadian isolates were, in general, highly virulent. Nine out of 12 isolates were virulent on more than half of the differential cultivars. The most virulent isolate among those applied to all 24 cultivars, was WRS1391 and the least virulent isolate was WRS1488 (Table 7). The relative virulence was determined by addition of the 8 binary numbers that make up the octal name and supported by comparing the number of compatible to incompatible reactions for each isolate.

We reduced the differential set to eight cultivars for the final two screenings. The eight cultivars chosen were of the most interest to our study as they carried the most well defined resistance genes. The reduction of cultivars also allowed inoculation of more isolates at a time as growth cabinet space was limited and time was a factor. Dendrogram results using these eight cultivars are similar to the cluster based on 24 cultivars with the exception of isolates WRS1426 and WRS1493 which move from the bottom group to the top (Figure 2). The two clusters are also less than 50% similar indicating a greater difference between the groups.

Table 7 Comparison of virulence among *Rhynchosporium secalis* isolates based on the total of the binary numbers within an octal number for each isolate and the number of compatible reactions on 24 differential cultivars.

Isolate	Octal total	compatible/incompatible
WRS1391	45	19 / 5
WRS1426	40	15 / 8
WRS1402	37	15 / 9
WRS1483	36	17 / 7
WRS1380	36	15 / 9
WRS1784	32	14 / 10
WRS1474	32	14 / 10
WRS1389	30	14 / 10
WRS837	29	11 / 13
WRS1780	26	13 / 11
WRS1493	23	11 / 13
WRS1488	22	10 / 14

Figure 2. Dendrogram of *Rhynchosporium secalis* isolates based on similarity of virulence on 8 barley cultivars.


0.0
0.25
0.50
0.75
1.00
1391
1380
1784
1860
1824
1483
1389
1474
1780
1488
1493
837
1859
1426
1402

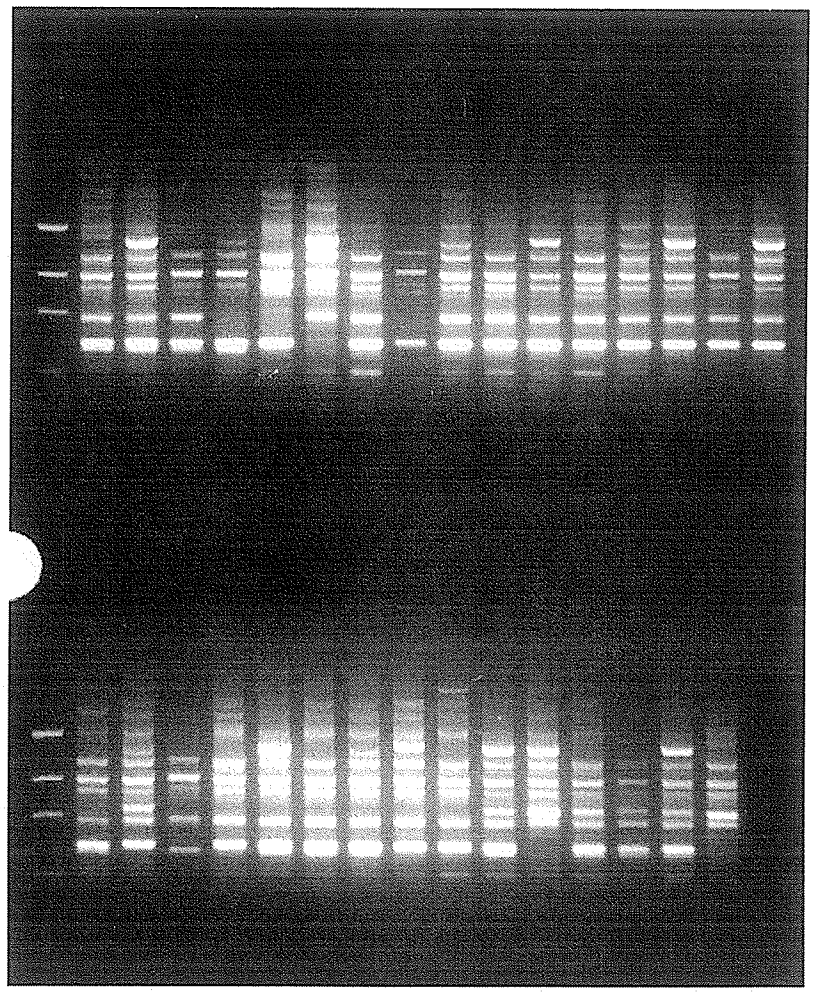
4.2 RAPD results

DNA extractions were done on *Rhynchosporium secalis* cultures that had the most growth in Fries medium at the time of extraction. Two isolates, WRS1391 and WRS1488, that were representative of the two main clusters in terms of virulence were chosen for initial screening with RAPD primers. Only those primers that identified a difference between these two isolates were applied to the remainder of the isolates. In total, 320 arbitrary sequence 10 base primers were applied. 157 of these primers amplified fragments in both isolates and 80 resulted in the amplification of polymorphic fragments. These 80 primers were applied to an additional 6 isolates which amplified 477 fragments, approximately 50% of which were polymorphic between at least two of the isolates. Thirty-three of the primers, selected based on band clarity, were applied to four more isolates. Across all 12 isolates, 300 different fragments were amplified and 118 were polymorphic. To evaluate the level of heterogeneity found among the Canadian *R. secalis* isolates in a broader context, a subset of twenty-seven of the original primers were applied to a total of 17 Canadian isolates in addition to 14 non-Canadian isolates from Australia (AU), Germany (CV), the United Kingdom (UK) and the United States (US, MT, and FF). The 27 primers generated 72 polymorphic fragments. 92-BSMV#98 was not included in the final amplifications as the stock of DNA was depleted and a pure culture could not be raised for another extraction. There was an average of 3 polymorphisms per primer (Figure 3). Polymorphic bands were scored as a '1' if strongly present and as a '0' if absent. A band that was monomorphic across all

Figure 3. Agarose gel showing results of RAPD primer OPD 20 across 31 *Rhynchosporium secalis* isolates.

top lanes 1-17: lambda marker, isolates WRS1391, WRS1389, WRS1474, WRS1780, WRS1784, WRS1488, WRS1380, WRS1426, WRS1483, WRS1402, WRS1493, WRS837, WRS1824, WRS1859, WRS1860, WRS1862.

bottom lanes 18-33: lambda marker, isolates WRS1864, MT92, FF94, CV1, CV3, UK5, UK7, UK8, AU1, AU2, AU3, AU4, AU5, US238, US262.



isolates, or was weakly amplified was not included. A similarity matrix was contructed in NTSYS using the polymorphism data collected in the final screening of RAPD primers on all 31 *R. secalis* isolates analyzed. The similarity matrix was then used as the basis for the production of a dendrogram (Figure 4). The dendrogram shows the similarity among isolates based on DNA sequence. The isolates are spread throughout the tree with only a slight correlation to geographic origin. The level of heterogeneity among Canadian isolates appears to be similar to the level of variation found among non-Canadian isolates with the exception of the Australian isolates. The Australian isolates tended to group together, which could be an indication of their geographic isolation and potentially separate evolution. Subsets of isolates were analyzed based on geographic origin for comparison with the virulence data of Canadian isolates (Figure 5). The two groupings of isolates seen in the dendrogram based on virulence are not present in the clustering based on RAPD analysis. Removing the non-Canadian isolates did not dramatically alter the order of the cluster, but it did show that the Australian isolates are as similar to one another as to other isolates. Many different geographic combinations of the data were analyzed by SAHN clustering and the results were consistently the same as above (Figure 6).

4.3 NIP 1 results

Dr. Wolfgang Knogge (Max Planck Institut, Köln, Germany) designed the NIP 1 primers based on the sequence of isolate US238.1. These primers were applied

Figure 4. Dendrogram based on similarity of RAPD fragment polymorphism patterns of 31 *Rhynchosporium secalis* isolates.

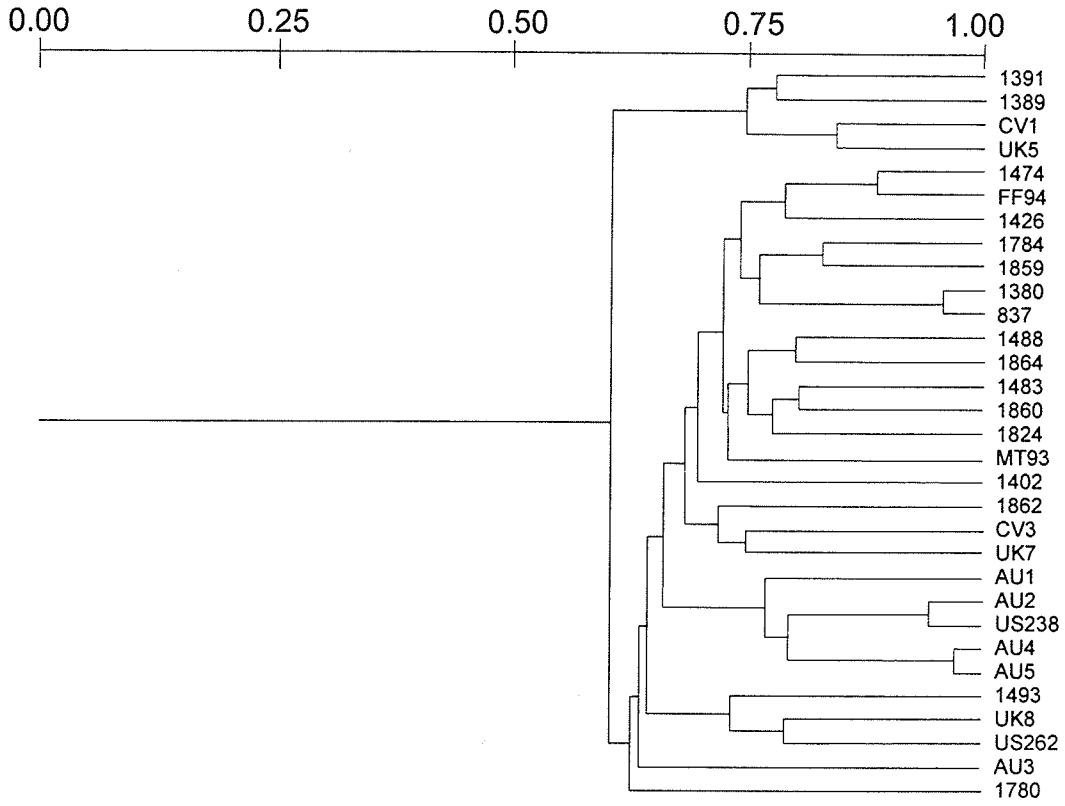

0.00
0.25
0.50
0.75
1.00
1391
1389
CV1
UK5
1474
FF94
1426
1784
1859
1380
837
1488
1864
1483
1860
1824
MT93
1402
1862
CV3
UK7
AU1
AU2
US238
AU4
AU5
1493
UK8
US262
AU3
1780

Figure 5. Dendrogram based on the similarity of RAPD polymorphic fragments of Canadian *Rhynchosporium secalis* isolates.

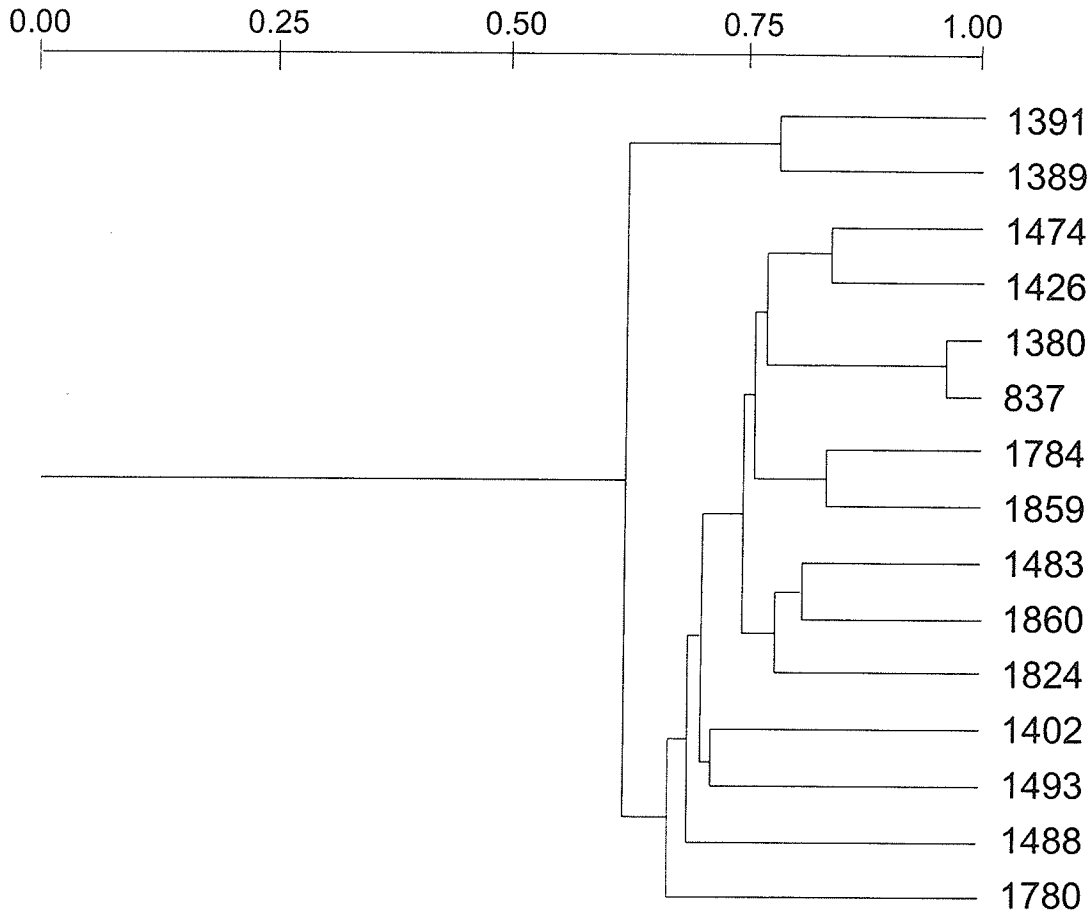
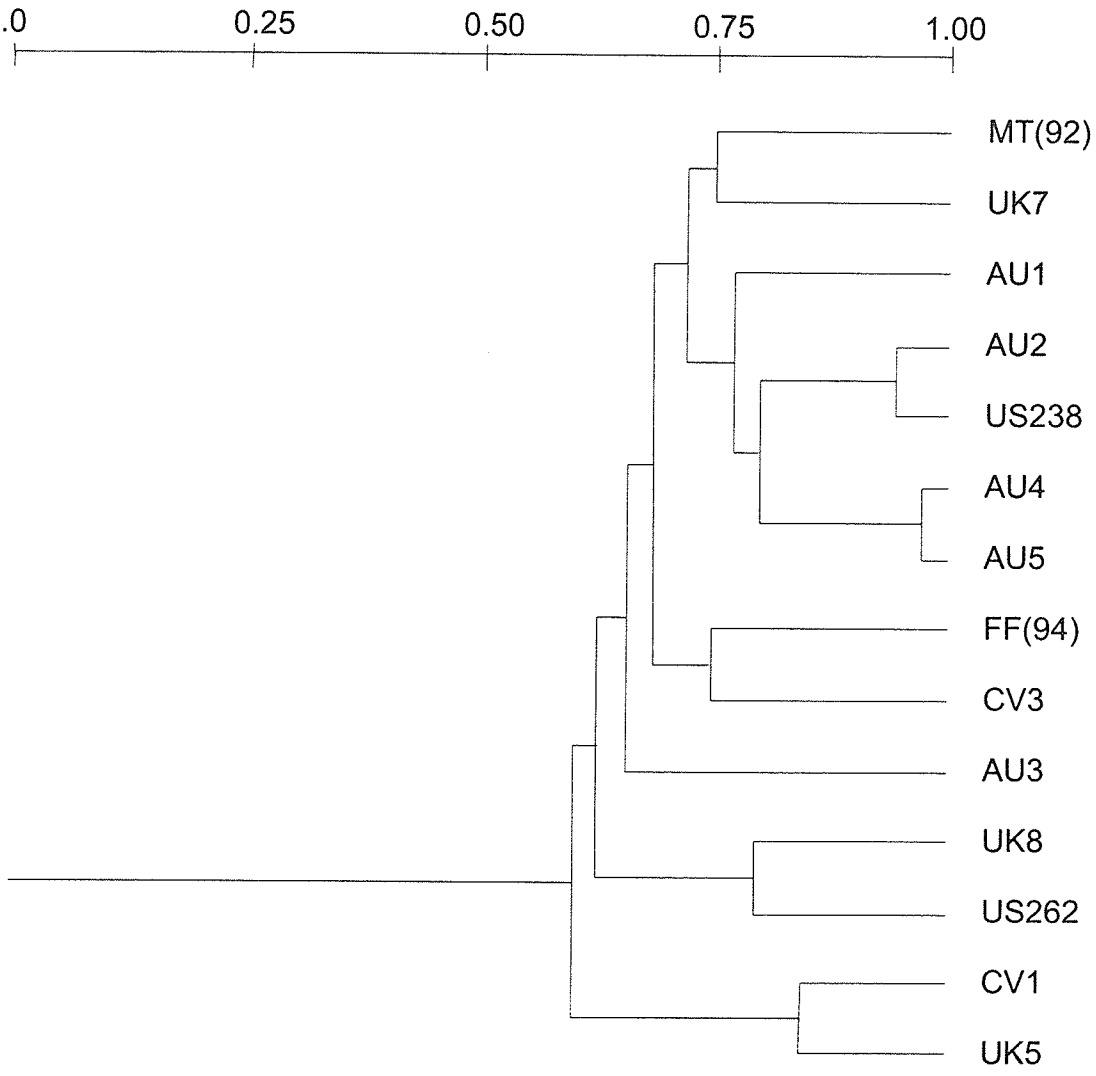

0.00
0.25
0.50
0.75
1.00
1391
1389
1474
1426
1380
837
1784
1859
1483
1860
1824
1402
1493
1488
1780

Figure 6. Dendrogram based on similarity of RAPD polymorphic fragments on non-Canadian *Rhynchosporium secalis* isolates.


0.0
0.25
0.50
0.75
1.00
MT(92)
UK7
AU1
AU2
US238
AU4
AU5
FF(94)
CV3
AU3
UK8
US262
CV1
UK5

to the DNA of all of our isolates and it was found that all the Canadian isolates carry the NIP 1 gene except for WRS1860 (Figure 7). Sequencing reactions were carried out with isolates WRS1389, WRS1860 (x2), WRS1391, WRS837, WRS1493, WRS1824, and WRS1864. Using the NIP 1 primers, the entire reading frame and intron of the NIP 1 gene from these isolates was sequenced (Figure 8).

To sequence an allele of the NIP 1 locus from WRS1860, the pipette poke method was used at the site where the band should have been and re-amplified what was present in the gel at that position. The second amplification resulted in a band of appropriate size for the NIP 1 gene that could be sequenced. It is possible that the first amplification did not yield enough product to be seen on the agarose gel and a second amplification sufficiently increased the yield to detect a product. WRS1860 had the same DNA and amino acid sequence as WRS1864, WRS1824, WRS1493, and WRS837 even though the NIP 1 gene of WRS1860 did not visibly amplify with primers 3 and 4 while the others did.

Comparison of NIP 1 gene sequences from the scald isolates at the primer binding site was impossible, as it is conserved from the original primers throughout amplification. There were, however other sequence differences that were significant as they coded for a change in amino acid sequence (Figure 9). The nucleotide and amino acid sequences of isolate WRS1389 were similar to that of WRS1391, the only difference being at nucleotide 302 which is the last base of the intron. We also found WRS1860 and AU2 to be quite similar, with a single difference being at nucleotide 199 that codes for glycine in WRS1860 and arginine in AU2.

Figure 7. Amplification of NIP 1 fragment with specific primers on 29 *Rhynchosporium secalis* isolates.

top lanes 1-16: lambda marker, WRS1391, WRS1389, WRS1474, WRS1780, WRS1784, WRS1488, WRS1380, WRS1426, WRS1483, WRS1402, WRS1493, WRS837, WRS1824, WRS1859, WRS1860.

bottom lanes 17-31: lambda marker, WRS1862, WRS1864, MT92, FF94, CV1, CV3, UK5, UK7, UK8, AU1, AU2, AU3, AU4, AU5.

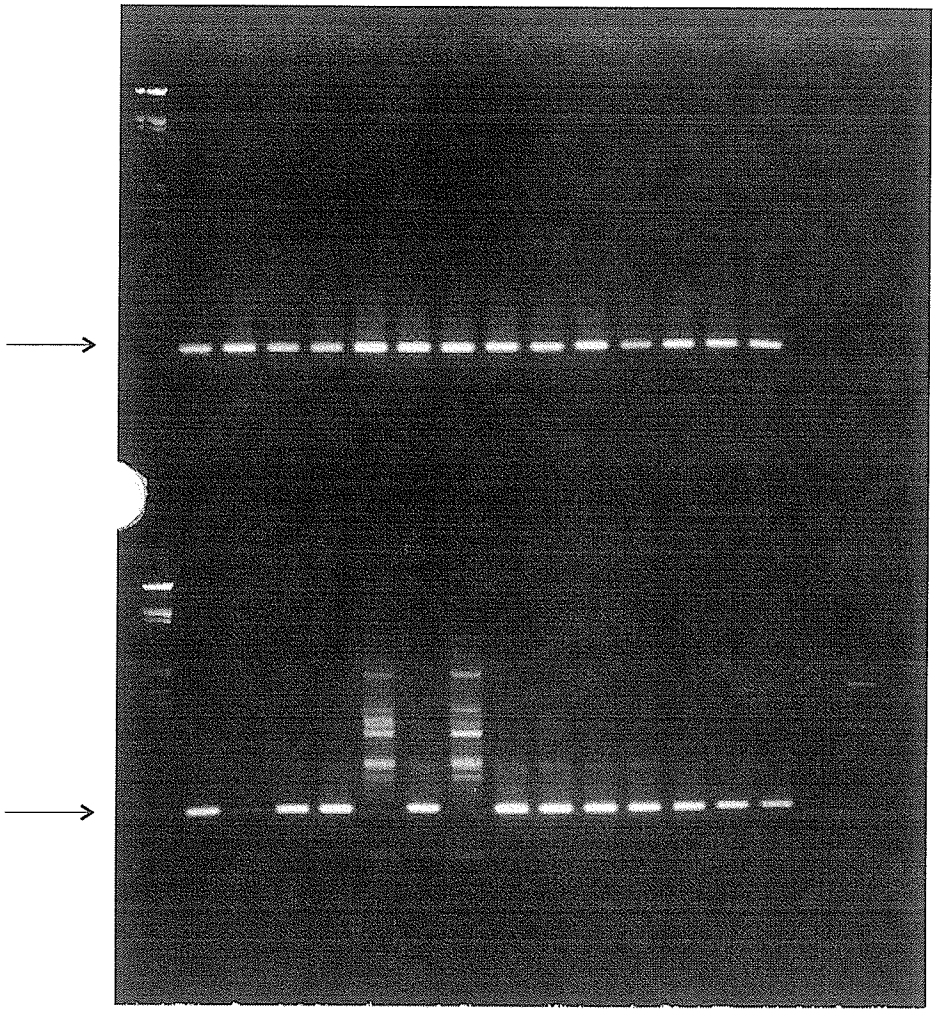


Figure 8 **NIP 1 sequence (US238)**

The start and stop codons are underlined, the intron is marked with the dotted underline and the NIP 1 primers are double underlined. The putative TATA box is in bold print.

US238 **-240**
ATCCTCAGCTTCTGTGAGTATACTTGGTTTCCAGGATAATTAAGCCATTGCCATCCACA
-180
GGGATCCTGATAGAACTATTCCCTGCAGTAACAAGTTCTATCAGGGTCGACCGTCTC**TAT**
-120
ATAATCAGTCTTCCTCCTCAACCTGCTCATCACATACCAGTCTTTGATTCCCAAACAAAA
-60
TTTTGCCACTTCTGCTTCTCCGAAAAAGAATACCCTTTAACCTCCAACTGAACCAAAACT
1
ATGAAATTCCTCGTACTGCCTCTCTCCCTGCCTTTCTTCAGATTGGCCTCGTCTTCTCT
61
ACGCCGGATCGATGCAGATACACCCTTTGTTGCGATGGAGCTCTCAAAGCTGTTTCCGCA
1860 a
1389 a
1391 a
AU2 a
121
TGCCTACATGAGTCAGAATCCTGCTTGGTTCCTGGGGATTGTTGCCGGGGAAAGTCCCGT
1860 a a t
1389 c t c
1391 c t c
AU2 a a t
181
TTGACGCTTTGCTCATATGGTGAAGGTGGCAATGGCTTCCAATGCCCGACGGGATACGTA
1860 g a
1389 g c
1391 g c
AU2 c a
241
AGTTTATTCATATCCGGAAAACCGTAGCTACAGGCACTCTCTAACAAAACGTTCAAATGT
301
AGCGCCAATGTTAAGGAAGAACTGTGCATCGCGCATATCCCTGCTTGAGCGACTAGCTGA
g 1860
g 1389
a 1391
g AU2
361
TAGGCGAACGGATCACTCCAAAGGGTCTCGCTCTAGCTGAGAATTGACAAAGAAAGAAAT
421
GAAACATGACATTGAGCTAAACACTGAAATACATCGGTGCATCAATTTAAATGTCATGCC
481
TTTCAGGGACGCGGGACCTGGACCCACTCTGTGCTGCTACATGAACTAAATCTGAACCATT
541
GTCCCTGCCATATAGCAATAATGCAATTCATATACTATCTATCGTGGCGGCTTT **595**

Figure 9 NIP 1 amino acid sequence

US238 MKFLVLPLSLAFLQIGLVFSTP**DRCRYTLC** 30

US238 **CDGALKAVSACLHESESCLVPGDCCRGKSR** 60

1860	E	Q	V
1389	E	P	A
1391	E	P	A
AU2	E	Q	V

US238 **LTLCSYGEGGNGFQ**CPTGYRQC 82

1860	G	K
1389	G	T
1391	G	T
AU2	C	K

Amino acids in bold are those found by sequencing of the purified protein and in normal print are those amino acids that comprise the transfer peptide and C-terminal.

There is a gene-for-gene relationship between the *Rh3* gene of Atlas 46 and the NIP 1 gene of *Rhynchosporium secalis*. Atlas 46 was derived by backcrossing the *Rh3* gene from Turk into Atlas. Scald isolates that are avirulent on Atlas 46 due to the *Rh3* resistance gene are also avirulent on Turk. Therefore, when NIP 1 is present in a scald isolate, that isolate should be avirulent on Atlas 46 but virulent on the near-isogenic cultivar, Atlas. The above situation held true, except for AU2 where the NIP 1 gene is present but not expressed and a virulent reaction results (Rohe et al, 1995). Amplification of the non-Canadian isolates showed that CV1 and UK5 lack the NIP 1 gene although UK8 should also be missing the NIP 1 band based on previous results (Rohe et al, 1995).

4.4 NIP 2 results

Degenerate primers were designed from the amino acid sequence of NIP 2 and applied to the *Rhynchosporium secalis* genome. Amplification with the NIP 2 degenerate primers resulted in many fragments being amplified. A band of the appropriate size was isolated and sequenced. The first attempts at sequencing demonstrated that only the forward primer was amplifying and that there were either multiple priming sites or more than one template present in the mixture. These findings led to optimization of the primer concentrations at a 1:4 ratio of forward to reverse primer. At the optimized concentration, the primers amplified the appropriately sized fragment more efficiently. The adjusted primer ratio was used to sequence this fragment (Figure 10). Specific primers were designed based on

Figure 10 a) NIP 2 DNA sequence. Underlined bases show the position of the specific primer binding sites

b) NIP 2 amino acid sequence

a) TATNGGGGANGTGGAGTGGGCAATTCAAAATAGGCGTCATGATTTAGCCCTTGGTGG

AAAAGGATTCTGGAGAGGGCATTCAACGTCTTGTCACAGGAACGCAAACGCCGTCGTCGA

CGT CGTC 124

b) provided by Dr Knogge Y Y V V V - V P R D G A E I G D V E W A A I Q N R
from DNA sequence M G E V E W A A I Q N R

R H D L A L G G K G F W R G H S T S C H R N A N A V V D V V A L 56
R H D L A L G G K G F W R G H S T S C H R N A N A V V D V V 41

the sequence data, and applied to all of the isolates from which we had DNA. All of the Canadian and non-Canadian isolates carried the NIP 2 gene band (Figure 11). A search of the Blast server found no matches to the NIP 2 sequence.

4.5 NIP 3 results

Degenerate primers for amplification of NIP 3 were designed from the amino acid sequence of NIP 3 (Figure 12) along with a consensus of codon usage from other fungi and from the NIP 1 amino acid and nucleotide sequence comparison. Application of these primers indicated that all of the isolates have the NIP 3 gene (Figure 13). These fragments were not sequenced.

Figure 11. Amplification of NIP 2 fragment with specific primers applied to 29 *Rhynchosporium secalis* isolates.

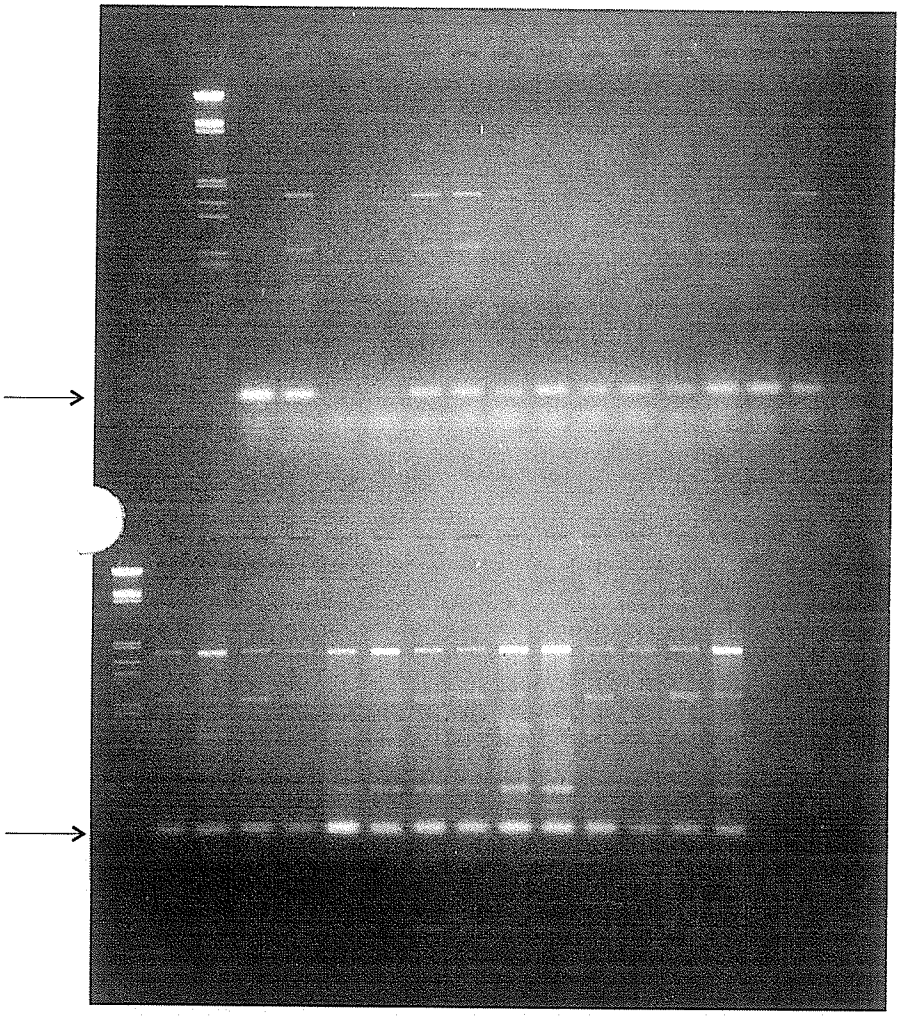
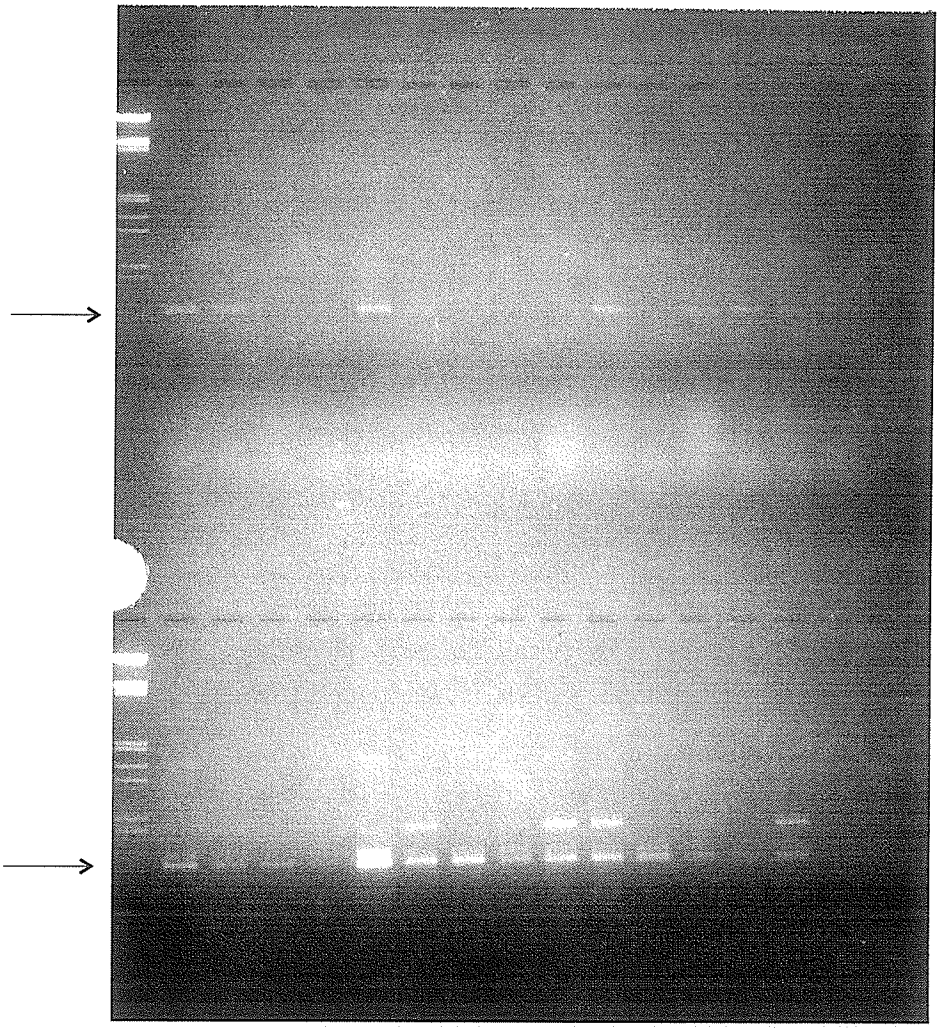


Figure 12 Amino acid sequence of NIP 3

A-GDVYTIKG--TIRDYK-KXXVPS-NSKG

A-PYLHLFDVPIFFDQDKKDVVSYK 55

Figure 13. Amplification of NIP 3 fragment using primers derived from the amino acid sequence on 29 *Rhynchosporium secalis* isolates.



5. Discussion

5.1 Scald inoculation

Barley cultivars with increased numbers of resistance genes to *R. secalis* are continually being introduced as the demand for feed and malting barley crop production remains strong. Increased host resistance necessitates evolution of increased virulence by the pathogen for survival. The Canadian *Rhynchosporium secalis* isolates used to inoculate the barley differential cultivars were all highly virulent. Cross-contamination during inoculation and incubation in the growth chamber could also have caused increased levels of virulence. Measures were taken to avoid cross-contamination of isolates but controls such as partitions between inoculated plants could not completely prevent movement of spores (Goodwin et al, 1994). The virulence of an isolate may also change in culture and this is prone to happen if the isolate is not cycled through host plants on a regular basis (Goodwin et al, 1994). If a mutation resulted in greater virulence it would neither be selected for, nor against, as growth conditions are optimal so that the reduced fitness conferred by the increase in virulence is not detrimental.

None of the Canadian isolates had the same virulence spectrum on either the 24 or the reduced 8 cultivar differential set although, WRS1426 and WRS1483 exhibited the same virulence pattern on the 9 cultivars used by Tekauz in 1991. The cultivars in the subset of eight included in this study must contain a broader spectrum of resistance genes than that studied by Tekauz (1991). It seems that if enough cultivars are inoculated, almost every isolate collected could be

differentiated into its own pathotype. In Tekauz (1991), WRS1391 was the most virulent isolate, infecting seven of eight cultivars. Isolate WRS1391 was again the most virulent in this study, infecting nineteen of twenty-four cultivars. Isolates WRS1426 and WRS1483 infected just one cultivar besides the universal susceptible in Tekauz (1991) yet were among the most highly virulent of this study. Also, isolate WRS1493 was moderately virulent in Tekauz (1991) but only weakly virulent in this study which likely incorporates more resistance genes among the differential cultivars. The cluster analysis of virulence showed two clusters which could be an indication of two separate origins, migration events, or two different foundations of resistance.

The virulence of *Rhynchosporium secalis* is very heterogeneous and may vary within a single lesion (Brown, 1985) or change within two generations (Jackson and Webster, 1976b). *R. secalis* may have evolved on wild barley at a centre of diversity and recently spread throughout the world. Genetic diversity within a region would decrease as the spread of this pathogen was forced through bottlenecks caused by movement of a few isolates to new locations (Goodwin et al, 1993). The two virulence clusters among the Canadian isolates could represent movement of scald disease into Canada and a reduction of diversity to two major pathotypes. It is also possible that *R. secalis* was well adapted to infect native grass hosts worldwide and just recently became virulent on barley. This could result in increased levels of heterogeneity due to the differences of native grass hosts from which isolates originated. For example, there may have been two specific native

grass hosts in Canada which were responsible for the two types of virulence seen among *R. secalis* isolates on barley today. The latter is not likely as isozyme studies show that the same alleles are common to all scald populations (Goodwin et al, 1993), and the former is also doubtful as the levels of diversity found so far are quite high. However, multiple migration events into a single location would increase the genetic diversity (Goodwin et al, 1993). *R. secalis* isolates may have been brought to Canada in two different years or from two different locations resulting in two main sources of virulence. However, the two clusters of virulence seen in the dendrogram are probably a reaction to the two main sources of resistance genes in Canadian barley. The cultivar Hudson carries the *Rh* resistance gene and is a common ancestor for many current cultivars. Another common ancestor of Canadian barley is Atlas which has the *Rh2* resistance gene (Penner et al, 1996).

The octal nomenclature system was developed to simplify and standardize naming of *R. secalis* isolates (Goodwin et al, 1990). Assigning numerical names with this method was useful for determining whether isolates have similar virulence patterns and also shows the overall virulence of an isolate. The list of differential cultivars suggested by Goodwin et al (1990) included some cultivars which may not be helpful for most studies because their resistance is unknown or uncertain. These cultivars are the ones towards the bottom of the list (leftmost of octal number) and are probably the most suitable ones to be replaced by cultivars that are more relevant to an individual study. For example, the most commonly grown cultivars for a specific region, or new cultivars with potentially better resistance.

The inoculation of barley cultivars with *R. secalis* in this study did not help to clarify the genetic basis of resistance on the cultivars tested. However some recent work on the mapping of barley chromosome three has shown that there is a gene cluster or family that includes at least resistance gene *Rh* (Graner and Tekauz, 1996). Development of molecular markers that simplify identification of *R. secalis* resistance genes in barley is progressing. To date, molecular markers have been found for resistance genes *Rh* (Graner and Tekauz, 1996) , *Rh2* (Schweizer et al, 1995) and a gene that maps to the same region as the cluster on chromosome three (Barua et al, 1993). Genetic markers for plant traits such as disease resistance are tools that can increase the efficiency of breeding programs.

5.2 RAPD analysis

RAPD analysis of the *Rhynchosporium secalis* genome was performed in an attempt to characterize virulence and to determine whether the difference between the two virulence clusters was indicative of different populations. RAPDs are less costly than other molecular analysis techniques such as RFLPs and are faster and easier to apply (Williams et al, 1990). The entire RAPD procedure requires only one day to see results (after DNA extraction). Twenty-seven RAPD primers were applied to the *R. secalis* genome and 72 polymorphisms resulted. With an average of three polymorphisms per primer, it is likely that amplification was not restricted to certain regions, such as virulence genes, only. The level of genomic heterogeneity detected was not surprising given the high levels of variation

demonstrated for virulence and morphology by this fungus. The RAPD analysis results demonstrate that heterogeneity is not confined to specific areas of the genome but is spread throughout the entire genome, therefore it appears that the whole genome is highly mutable and not just the virulence genes. Isozyme studies have also shown variation at supposedly conserved gene sites for regulatory enzymes like β-glucosidase or glucose-6-phosphate isomerase (Newman, 1985, Goodwin et al, 1993).

The dendrograms based on similarity of the RAPD data between isolates did not demonstrate a relationship between genomic heterogeneity and the two virulence phenotype clusters noted earlier. A single base change can be the difference between amplification and non-amplification of a fragment. That is, one base change out of twenty bases (2 x 10 base primers) involved in primer recognition is able to cause a polymorphism. 36% of the total amplified bands were polymorphic which means that at least [(36 x 1) / 20] = 1.8% of the bases in the amplified regions are variable. The average genome size for fungi is 2×10^7 - 3.5×10^7 base pairs (Lewin, 1990). If the *R. secalis* genome is at the upper end of the range then 6.3×10^5 bases of the genome are variable. The high level of polymorphism detected with RAPD analysis combined with the number of loci assayed, suggests that the heterogeneity between *R. secalis* isolates is spread throughout the entire genome and cannot be confined only to regions associated with virulence/avirulence.

The virulence data suggested the presence of two populations of *R. secalis* in Canada and the RAPD data shows this to be unlikely. However, the small number

of isolates that were included in the study limit any interpretations that can be made about the population as a whole within Canada or worldwide. The ideal experimental situation for evolutionary conclusions requires a large number of isolates from a small region that are all collected from known cultivars with defined reistance genes.

5.3 Necrosis Inducing Peptides

Barley cultivars Atlas, Atlas 46 and Turk have reasonably well defined resistance to scald and carry the genes involved in the gene-for-gene interaction with NIP 1. Atlas contains the *Rh2* resistance gene (Dyck and Schaller, 1961) and Turk contains *Rh3* and *Rh5* (Dyck and Schaller, 1961). Atlas 46 was derived by backcrossing resistance to scald disease from Turk into Atlas and therefore has both *Rh2* and *Rh3* (Dyck and Schaller, 1961). Although these gene assignations are clear and well supported in both this study and others (Graner and Tekauz, 1996), there was additional resistance present in Atlas 46 that was not exhibited by either Atlas or Turk. The pedigree of Atlas 46 is Hanna / Atlas //// Turk (Aborg and Wiebe, 1946). It is possible that additional resistance was derived from Hanna or that the Turk accession used to derive Atlas 46 is not the same Turk accession widely used in subsequent genetic studies. It is also possible that the *Rh5* gene from Turk which was not backcrossed into Atlas 46 may impart susceptibility to certain isolates. Pathogen interactions with these cultivars are important because the *Rh3* gene is the resistance gene involved in the host specific reaction with the

NIP 1 gene product of *R. secalis* (Rohe et al, 1995). Rohe et al (1995) concluded that when the NIP 1 gene is present and active in the pathogen, it will elicit an avirulent reaction when it comes in contact with a host plant carrying the corresponding *Rh3* resistance gene. This follows the basic gene-for-gene hypothesis. Purified NIP 1 elicits non-specific stimulation of the plasma membrane H^+ ATPase and thereby acts as a toxin (Wevelsiep et al, 1993) and hence could be a potential virulence gene product. When NIP 1 is within the fungal cell, it is regulated such that it becomes a cultivar specific elicitor of host defenses (Rohe et al, 1995). The regulation may be in the transcription or translation of the gene product or during transport out of the fungal cell and into the host cell. To be a cultivar specific elicitor, the end product must also be in an active form with the correct signal peptides and conformation. If NIP 1 is not present or not active then there is no recognition event, a compatible (virulent) reaction occurs and the plant becomes infected. If the host plant does not carry the *Rh3* gene, again there is no recognition of NIP 1 and a compatible reaction occurs where NIP 1 may act as a toxin, provided that the pathogen carries no other avirulence genes that the host recognizes (Table 8). NIP 1 can cause cell collapse by stimulating the plasma membrane H^+ ATPase and changing the ion concentration in the plant cell (Wevelsiep et al, 1993).

There was a lack of agreement between the resistance phenotypes exhibited by Atlas, Atlas 46 and Turk, and the postulated genetic basis for resistance in these lines. The most reasonable explanation for the conflict between observed and

Table 8 Suspected interaction between NIP 1 from *Rhynchosporium secalis* and *Rh2* and *Rh3* genes from barley based on expected Host-Pathogen interactions between avirulence and resistance genes.

HOST

PATHOGEN	*Rh2*	*Rh3*
NIP 1	virulent	avirulent
Avr X	avirulent	virulent

expected resistance patterns is that the accession of Turk used in this study was not the same as the accession used in the derivation of Atlas 46. Based on this assumption, an excerpt from table 4 containing just Atlas and Atlas 46 was analyzed for host / isolate recognition to predict which isolates carry the NIP 1 gene (Table 9).

The attempted amplification of the NIP 1 gene with specific primers resulted in the generation of the appropriate size fragment from all the Canadian isolates with the exception of WRS1860. It follows that all the Canadian isolates, except WRS1860, should be avirulent on Atlas 46 and Turk. This was not the case as some isolates that exhibited the NIP 1 gene fragment also exhibited virulence on Atlas 46. Most of the isolates were avirulent to both cultivars and therefore probably carry the NIP 1 gene and/or another avirulence factor that is responsible for the resistance of Atlas and is recognized by the *Rh2* gene. Isolates WRS1784, WRS1483, WRS1493 and WRS1824 were virulent on Atlas but not Atlas 46 because NIP 1 was present and recognized by the *Rh3* resistance gene. Isolates WRS1860, WRS1391 and WRS1380 were virulent on both Atlas 46 and Atlas thus, implying that they lack NIP 1. To further investigate this apparent conflict, the NIP 1 gene from a number of isolates was sequenced. There are at least two possible explanations that could be invoked while maintaining the NIP 1 / *Rh3* gene-for-gene model. One, the NIP 1 gene in those isolates that did not give the expected results may differ in sequence such that they produce non-functional products. Two, the NIP 1 gene must not only be present and active but also be present in high enough

Table 9 Excerpt from table 4 with the observed virulence reactions of 15 isolates on Atlas and Atlas 46 and the expected virulence from molecular experiments.

		1391	1389	1474	605	77EM	1488	1380	1426	1483	1402	1493	837	1824	1859	1860
Atlas	obs	-	+	+	+	-	+	-	+	-	+	-	+	-	+	-
	exp	-	+/-	+/-	+/-	-	+/-	-	+/-	-	+/-	-	+/-	-	+/-	-
Atlas 46	obs	-	+	+	+	+	+	-	+	+	+	+	+	+	+	-
	exp	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-

+ is avirulence reaction, - is virulence reaction

concentrations to be recognized. Wevelsiep et al (1991) found that a much higher concentration of NIP 1 was required to cause necrosis than NIP 2 or 3. In the elicitor receptor model (Gabriel and Rolfe, 1990) of the gene-for-gene hypothesis a threshold concentration is required to trigger host defenses. The sequence differences between isolates could have an effect on the amount of NIP 1 that is produced and active. There were two isolates (WRS1493 and WRS837) that had the same sequence as WRS1860 but were avirulent on Atlas 46. WRS1860 may not have amplified due to a sequence difference in the primer annealing region that impeded primer binding. Enough product was generated however, such that a second round of amplification resulted in the production of the NIP 1 fragment. If the sequence difference that inhibits amplification is in the promoter region of the NIP 1 gene then perhaps WRS1860 was not produced in high enough concentrations to elicit the host defense response.

Another situation where the amplification results do not agree with the inoculation results was when an isolate was virulent on Atlas 46 and the NIP 1 gene was present. AU2 from Australia is one such isolate and it has been found that although the gene is present and a peptide is produced, it is not elicitor active (Rohe et al, 1995). The inactive protein could also be a result of sequence differences that cause amino acid changes to give a potential change in conformation rendering the peptide non-functional. A conformation change could leave the protein unable to exit the fungal cell or make it unrecognizable to the receptor protein on the host cell. Isolate WRS1389 which is avirulent on Atlas 46

and has NIP 1, has one base change in the amplified region from WRS1391 which is virulent and has NIP 1. The last base of the intron is different and could lead to mis-splicing of WRS1391's exons resulting in an inactive form of the protein.

The presence of the NIP 1 band on the gel could be used in most cases to predict avirulence on Atlas 46. It is possible that a primer based on the sequence variant exhibited by WRS1391 could be used to extend the predictability of this test. The correlation between the presence of a particular form of the NIP 1 gene and avirulence on Atlas 46 provides a useful tool for investigating population dynamics. It should be possible to track the evolution of virulence exhibited by both isolates WRS1860 and WRS1391 in western Canada in the future.

6. Conclusions

The virulence of *Rhynchosporium secalis* is highly variable and RAPD analysis of the genome suggests that heterogeneity is not confined to avirulence/virulence coding regions but is spread throughout the genome. The high level of genomic variability may be an indication of a high mutation rate which could bring about rapid changes in sequence. Any strategies for introduction of scald resistance to barley cultivars should therefore be assessed for effectiveness against a population with highly variable virulence.

The presence or absence of NIP 1 in the *R. secalis* genome can be used to predict the interaction of that isolate with barley cultivar Atlas 46. However, NIP 1 must also be functional to elicit an avirulence reaction with cultivar Atlas 46 and a single base change in the NIP 1 coding region can render the peptide inactive. Understanding of the specific NIP 1 interaction with *Rh3* is important as a first step towards understanding the *R. secalis* / barley interaction at the molecular level. A more complete picture of the genetic relationship between host and pathogen may help to determine a better method of disease control.

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