IDENTIFICATION OF EFFECTORS WITH AVIRULENCE FUNCTIONS IN THE PATHOGENIC BARLEY SMUT FUNGUS, USING MARKER-BASED APPROACHES AND COMPARISON AMONG GENOMES OF RELATED SPECIES

by

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ABSTRACT

In plant pathology, the molecular genetic analysis of the interaction between pathogen and host yields knowledge applicable to combat crop disease. During infection, pathogens secrete effector proteins to reprogram the host for its benefit. In special cases, recognition of certain effectors by resistance genes, essential components of the host surveillance system, induces resistance to infection. No effectors with such avirulence function have been described for basidiomycete fungi infecting cereals. *Ustilago hordei* is a biotrophic basidiomycete fungus that infects barley. One of its effectors functions as an avirulence protein, UhAVR1, rendering it avirulent on barley cultivar Hannchen, having corresponding resistance gene *Ruh1*. I have located *UhAvr1* within the genome using a deletion approach and confirmed its resistance-triggering function. I provide evidence that transposable element (TE) activity in the *UhAvr1* promoter region and translocation of the coding region are likely responsible for enabling virulence on Hannchen. This region of the genome harbours a cluster of predicted secreted proteins and is syntenic to a cluster in closely-related corn pathogens, *U. maydis* and *Sporisorium reilianum.* In *U. maydis*, deletion of this region results in dramatic reduction in virulence on corn. This region is under selection pressure in both *U. maydis* and *U. hordei* likely to avoid recognition by the host. Evolution of the region in *U. maydis* seems to involve gene duplication and diversification, while in *U. hordei* this region is saturated with TEs and repeats which can play a role in genome rearrangements.

Computational analysis of the *U. hordei* genome sequence identified 372 candidate secreted effector proteins (CSEPs), many of which are expected to contribute to virulence and some to trigger resistance in analogy to UhAVR1. Most CSEPs are *Ustilago*-specific proteins of unknown function and without similarities to sequences in public databases. Evidence for accelerated evolution was observed when comparing CSEPs among smut species. More than half of these CSEPs have four or more cysteine residues in characteristic patterns, possibly involved in disulphide bridge formation and protein folding.

The study of effectors with avirulence function can reveal resistance genes which can be used for crop breeding programs to obtain disease-resistant cultivars.

PREFACE

Below is a list of manuscripts that have been published or are in preparation for publication and that comprise this thesis. The contributions made by the candidate are mentioned.

Chapter 1: A shortened version of the chapter will be submitted as review for publication. Anticipated author: Ali, S. and Bakkeren, G.

The candidate wrote the chapter. Dr. Bakkeren supervised the manuscript preparation and provided editorial support.

Chapter 2: A version of the chapter entitled "The avirulence gene *UhAvr1* clusters with predicted secreted proteins in the *U. hordei* genome and is inactivated by transposon activity in virulent strains", is in preparation for publication. Anticipated co-author list: Ali, S., Linning, R., Laurie, J. and Bakkeren, G.

The candidate designed and performed the experiments, as well as wrote the manuscript. Mr. Linning assisted with the preparation of Figures 2.12 and 2.16 and provided bioinformatic support. Dr. Bakkeren supervised the work and manuscript preparation and provided editorial support.

Chapter 3: A version of this chapter will be included in a joint manuscript entitled "Genome-wide analysis of *U. hordei* candidate secreted effectors proteins; comparison with *U. maydis*." which is in preparation for publication. Anticipated co-author list: Laurie, J., Ali, S., Linning, R., Bakkeren G., Schirawski, J., Kahmann, R. and others.

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Chapter 4: This work was published in its entirety in Current Genetics, 57: 63-73, Ali, S. and Bakkeren, G. 2011. ”Introduction of large DNA inserts into the barley pathogenic fungus, *Ustilago hordei*, via recombined binary BAC vectors and *Agrobacterium*-mediated transformation.” .

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The candidate designed and performed the experiments, as well as wrote the chapter. Dr. Bakkeren supervised the work and manuscript preparation and provided editorial support.

Chapter 6: “General discussion.”

The candidate wrote the chapter. Dr. Bakkeren provided editorial support.

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aa	Amino acid
ABA	Abscisic acid
AD	Activation domain
AFLP	Amplified fragment length polymorphisms
AMT	*Agrobacterium* mediated transformation
AS	Acetosyringone
Avr	Avirulence
BAC	Bacteria artificial chromosome
BD	Binding domain
Bgh	*Blumeria graminis* f. sp. *hordei*
BIBAC	Binary BAC
BL	Left border
BR	Right border
BSA	Bulked segregant analysis
CAT	Chloramphenicol acetyl transferase
CC-NBS-LRR	Coiled-coil nucleotide-binding site, leucine-rich repeat
cDNA	Complementary DNA
CF	*Cladosporium fulvum*
Cm	Chloramphenicol
CM	Complete medium
CSEPs	Candidate predicted secreted proteins
CSP	Cold shock protein
DAMPs	Danger associated molecular patterns
DCM	Double complete medium
DCM-S	Double complete medium-sorbitol
ECL	Enhanced chemiluminescence system
ECP	Extracellular cysteine-rich proteins
Ef-Tu	Elongation factor Tu
ETI	Effector-triggered immunity
Fol	*Fusarium oxysporum* f. sp. *lycopersi*
GFP	Green fluorescent protein
GUS	Bacterial *uidA* gene (beta-glucuronidase)
HESP	Haustoria-expressed secreted proteins
HR	Hypersensitive response
HTS	Host targeting signal
Hyg B	Hygromycin B
IM	Induction medium
JA	Jasmonic acid
Kb	Kilo base
Km	Kanamycin
LB	Luria-Bertani
LPS	Lipopolysaccharides

LTR	Long terminal repeat
MAMP	Microbe associated molecular
MAT-1	Mating type 1
MAT-2	Mating type 2
mg	Millegram
Mig	Maize induced gene
Ml	Mildew
ml^{-1}	Per milleliter
MPa	Mega pascal
Nd	Niederzenze
ng	Nanogram
NJ	Neighbor-joining
NLP	NEP1-like proteins
NLS	Nuclear localization signals
NO	Nitric oxide
NPSPCs	Non-predicted secreted paralogs of CSEPs
NRPS	Nonribosomal peptide synthetase
^oC	Degree Celsius
ORF	Open reading frame
PAL	Phenylalanine ammonia lyase
PAMP	Pathogens associated molecular pattern
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PEG	Polyethylene glycol
%	Percent
PKS	Polyketide synthase
PR	Pathogenesis-related
PRR	PAMP recognition receptors
Psi	Pounds per square inch
Pst	*Psedomonas syringae* pv tomato
PTI	PAMP triggered immunity
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
R	Resistance
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphisms
RIP	Repeat-induced point
RLK	Receptor-like kinases
ROS	Reactive oxygen species
Ruh1	Resistance to *Ustilago hordei Avr1*
RXLR	Arginine any amino acid leucine arginine
SA	Salicylic acid
SCAR	Sequence-characterized amplified region
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIMAP	Similarity matrix of proteins

SNP	Single nucleotide polymorphism
SSR	Simple sequence repeat
SP	Signal peptide
SSPs	Small secreted proteins
SUMO	Small ubiqiuitin-like modifier
TBE	Tris-borate-EDTA
Tc	Tetracycline
T-DNA	Transfer DNA
TE	Transposable element
Ti	Tumor inducing
TILLING	Targeting induced local lesions IN genomes
TIR	Toll or interleukin 1 receptor
TMD	Transmembrane domain
TTSS	Type three secretion systems
UAS	Upstream activating sequences
UhAvr1	*Ustilago hordei Avr1*
UPA	Upregulated by AVRBS3
v/v)	Volume/volume
w/v	Weight/volume
w/w	Weight/weight
WsB	Wassilewskkija
Xcc	*Xanthomonas campestris* pv *campestris*
YXC	Tyrosine any amino acid cysteine
Zeo	Zeocin
μg	Microgram
μl	Microliter

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CHAPTER 1

Introduction

1.1. Plant-microbe interactions

Plant diseases cause large crop losses worldwide. Approximately 14.1% of total crop yields are destroyed by diseases caused by both biotic and abiotic sources (Agrios 2005). Plants are attacked by many pathogenic organisms including fungi, oomycetes, bacteria, viruses, aphids and nematodes. Fungi cause the most serious damage among all plant pathogens, with an estimated annual loss of 200 billion $US (Horbach, et al. 2011), and account for 12 of the 19 most serious pathogens in the US (Madden and Wheelis 2003). There are more than 100,000 species of fungi, and more than 10% of them are plant pathogens, while only 50 fungal species are human pathogens and a similar number are animal pathogens (Agrios 2005). In North America, more than 8,000 fungal species are plant pathogens that cause more than 100,000 diseases (Horbach, et al. 2011).

By definition, plant pathogens are organisms that have the ability to cause disease on plants; however, it is widely accepted that plants are resistant to most pathogens, which makes plant disease the exception rather than the rule. Such pathogens are called non-adapted or nonhost pathogens and the plants are called nonhost plants. When a pathogen can infect one or more members of a plant species, the pathogens are called adapted or host pathogens and the plant is called a host for that particular pathogen. Phytopathogens can be divided into three classes: necrotrophs, biotrophs, and hemibiotrophs, based on their lifestyles and mode of interaction with their hosts. Necrotrophs kill cells of their host plants often through the production of toxins at the first attempt of colonization and subsequently feed on dead cells (Horbach, et al. 2011). Besides toxins, necrotophs also elicit cell death by secreting reactive oxygen species (ROS) and large amounts of cell wall degrading enzymes that also causes significant tissue damage (Rohe, et al. 1995, Tudzynski and Kokkelink 2009, Walton 1994).

A shortened version of the chapter will be submitted as a review for publication. Anticipated author: Ali, S. and Bakkeren, G.

Some of the host plants have developed abilities to recognize the secreted toxins at early infection stages of necrotrophs; thus, these weapons become liabilities for the pathogens (Rohe, et al. 1995). Biotrophs do not produce toxic secondary metabolite or toxic proteins since they need living host cells to supply the nutrients for long periods of time to complete their life cycle (Dangl and Jones 2001). The obligate biotroph has evolved a mechanism to suppress the host defense response or to avoid recognition during infection of living tissues (Greenberg 1997); however, some host plants can recognize pathogen-specific molecules which then trigger rapid and localized cell death that can stop pathogen growth to the neighboring cells, a the so-called, hypersensitive response (HR). Hemibiotrophs combine these two lifestyles by acting as biotrophs in the beginning of the infection to avoid detection and multiply in association with living cells, and then as necrotrophs, by killing their host cells toward the end prior to dispersal (Horbach, et al. 2011).

1.1.1. Strategies used by pathogens to infect host plants

For a pathogen to infect a plant, it first needs to recognize its plant host. Pathogens use different strategies to enter their host plants. For example, viruses use other organisms as vectors to enter plant cells and proliferate intracellularly, while pathogenic bacteria enter plants through natural openings normally used for gas and water (stomata and hydathods, respectively) or through wounds; they proliferate in the apoplast. Fungi and oomycetes enter either through natural openings or directly through the plant epidermal cells by mechanical and chemical means, or expand their hyphae on the top of, within, or between the plant cells (Jones and Dangl 2006). The pathogens need to adhere to the plant before penetrating the plant cuticle. Fungal hyphae and spores use mucilaginous and adhesive substances at their tips and the intermolecular forces between plant and pathogen are responsible for the close contact. Most rust fungi enter plants through stomata by developing appressoria over the stomata to penetrate into the cavity below, while ascomycetes such as *Magnaporthea grisea* and the powdery mildews, such as *Blumeria graminis* f. sp. *hordei* (*Bgh*) penetrate the cuticle of plants directly through appressorium. For direct cuticle penetration, *M. grisea* uses turgor pressure in its melanized appressorium (which is about 8 MPa) for penetration while *B. graminis* f. sp. *hordei* penetrates cell walls by the combined activity of cellulases and a turgor pressure of about 2-4 MPa (Pryce-Jones, et al. 1999, Talbot 2003). Other pathogens secrete cell wall degrading enzymes, such as cutinases, cellulases,

pectinases, and wax degrading enzymes for penetration of their host. After gaining entrance to the host plants, pathogens require additional "weapons" to neutralize the defense reaction of the host and gain access to nutrients.

1.1.2. Resistance in plants to pathogens

Resistance to most non-adapted pathogenic microbes is achieved by preformed physical barriers, such as waxy cuticular surface layers of the leaves that prevent pathogens from entering into the plant. Even after successful penetration of the cuticle, the pathogen needs to overcome the biochemical barriers that include low pH of the apoplast, broad spectrum antimicrobial compounds, and "defense" enzymes that degrade microbial cell walls (Felle 1998, Dangl and Jones 2001, Huckelhoven 2007). In addition, plants cells are surrounded by thick and stable cell walls that most microbes are not equipped to penetrate. The cell wall components such as actin microfilaments play an important role in defense against fungal penetration and whose disruption results in loss of nonhost resistance against several nonhost fungi (Kobayashi, et al. 1997a, Kobayashi, et al. 1997b, Mysore and Ryu 2004). Plants also produce a number of peptides, proteins, and non-proteinaceous secondary antimicrobial metabolites which may determine the host range of some pathogens (Broekaert, et al. 1995, Morrissey and Osbourn 1999). Other plants like tomato and sugar beet produce fungitoxic substances on the surface of leaves to inhibit the germination of spores of *Botrytis* and *Cercospora,* respectively (Agrios 2005). Similarly, some red scale varieties of onion contain the phenolic compounds, protocatecuic acid and catechol, which inhibit the germination of conidia causing them to burst, thereby protecting the onion plant from infection (Agrios 2005). Phytoanticipins (the preformed antimicrobial compounds) like tannins and fatty acid-like compounds such as dienes, present in high concentration in cells of young fruits, leaves, or seeds are responsible for resistance to pathogens such as *Botrytis* (Agrios 2005). Saponins, another group of phytoanticipins, such as tomatine in tomato and avenacin in oat, provide resistance to *Septoria* and *Gaeumannomyces graminis* var. *avena,* respectively, when the pathogens lack the corresponding detoxifying enzymes, such as saponinase (Osbourn 1996). Protease inhibitors are another class of antimicrobial compound which were originally isolated for their anti-feedant activities against insects. Reports have shown that proteinase inhibitors such as cysteine protease inhibitor produced by pearl millet have anti-fungal activities (Joshi, et al. 1999). Tomato and potato produce several cysteine proteases

and secrete them to the apoplast to control pathogenic bacteria, fungi, and oomycetes (Axtell and Staskawicz 2003, Lucas 1998).

Besides preformed defense systems, plants also have inducible defense systems to produce antimicrobial compounds such as the phytoalexins. Pea produces pisatin when attacked by *Nectria* spp. and rice produces sakuranetin against *M. grisea* (Agrios 2005). Pathogenesis-related (PR) proteins are defense proteins that are induced in response to pathogen attack. The accumulation of these proteins is usually associated with the acquisition of systemic resistance in plants against a wide range of pathogens (van Kan, et al. 1992). The PR proteins produced in plants are chitinases, phenylalanine ammonia lyase (PAL), β -1, 3 glucanase, PR-1, PR-4 to PR-14, and peroxidase (Agrios 2005). In tobacco, five groups of PR proteins have been identified, each group consisting of an acidic, extracellular and basic, intracellular protein (van Loon, et al. 1987). PAL is one of the key enzymes involved in the synthesis of aromatic compounds, like phytoalexins which are involved in stress and disease resistance (Dixon and Harrison 1990). Chitinase and β-1,3 glucanase are capable of hydrolyzing the chitin and β-1,3 glucans, which are two major polysaccharides of the cell walls of many pathogenic fungi (Kauffmann, et al. 1987, Legrand, et al. 1987). In addition to induced chemical defenses, plants also use induced physical defenses against pathogens, for example, by forming cell wall depositions (papilla) directly under the penetration sites of the pathogens; this can stop up to 90% of the penetration attempts in the *B. graminis* f. sp. *hordei /Arabidopsis* nonhost interaction (Collins, et al. 2003).

Some physiological responses seen upon incompatible interactions are the increase in Ca^{2+} levels in the cells surrounding the recognition site, which is the first measurable defense response and is required for the HR (Grant and Loake 2000). The oxidative burst, the production of reactive oxygen species (ROS), is another early defense response. The accumulation of ROS is involved in cell wall cross-linking, up-regulation of defense gene expression, and induction of the HR (Torres and Dangl 2005, Torres, et al. 2006). Salicylic acid (SA), activates plant defense responses against invading pathogens (Durner, et al. 1997). SA is required for systemic defense in both host and nonhost resistance and is rapidly induced during the HR. Nitric oxide (NO) also acts as a signalling molecule and promotes the HR. The early physiological and biochemical events and signalling requirements in defense responses are almost similar in host, nonhost, and virulent pathogen interactions. The difference in the strength and timing of activation of these

defence pathways makes a resistant interaction different from a compatible interaction (da Cunha, et al. 2006).

1.1.3. Pathogen associated molecular patterns

In addition to preformed and inducible physical and biochemical barriers, plants also have surveillance systems that evolved to recognize various pathogen surface-exposed and cytoplasmic molecules known as pathogen (microbe) associated molecular patterns: PAMPs or MAMPs (Shiu and Bleecker 2003). MAMPs are highly conserved molecules of microbes and are perceived by host receptors called PAMP recognition receptors (PRRs) at an early stage of infection. Recognition results in induction of PAMP triggered immunity (PTI). Examples of surface-exposed PAMPs that have been shown to be capable of triggering PTI are flagellin (Felix, et al. 1999), lipopolysaccharides (LPS) (Erbs and Newman 2003, Meyer, et al. 2001), lipooligosaccharide from gram-negative bacteria, chitin from cell walls of higher fungi (Bartnicki-Garcia 1968, Ren and West 1992), invertase from *Saccharomyces cerevisiae* (Basse, et al. 1992), and 1,3-1,6-hepta-β-glucoside from the cell walls of *Phytophthora sojae* (Sharp, et al. 1984a, Sharp, et al. 1984b). Examples of cytoplasmic MAMPs that induce host defense are cold shock protein (CSP) and elongation factor Tu (Ef-Tu; (Felix and Boller 2003, Kunze, et al. 2004).

One efficient strategy for pathogens to avoid PAMP recognition and triggered host defense would be changing PAMP sequences that are recognized by the PRRs, but mutating PAMPs is not very easy due to their often essential roles for pathogen survival (Gohre and Robatzek 2008). One of the best characterized PAMPs is flagellin from gram-negative bacteria that is present in both animal and plant pathogenic bacteria. In *Pseudomonas syringae*, flg22, a highly-conserved peptide from the N-terminal part of flagellin, is a strong elicitor of defense in tomato and *Arabidopsis* (Felix, et al. 1999, Gomez-Gomez and Boller 2000); however, there are some examples such as in *Agrobacterium tumefaciens* and *Ralstonia solanacearum* where these bacteria have changed their flagellins in order to avoid recognition by corresponding PRRs (Felix, et al. 1999, Pfund, et al. 2004). Sun and co-workers also reported that isolates of *Xanthomonas campestris* pv *campestris* (*Xcc*) which causes black rot disease on crucifers, also show a high degree of variation in their flagellin sequences and that some flagellin variants had less elicitor activity than others without affecting the motility of bacteria (Sun, et al. 2006).

Lipopolysaccharides (LPS) from the cell wall of gram-negative bacteria are recognized by host plants as PAMPs and elicit the defense response (Dow, et al. 2000, Erbs and Newman 2003, Meyer, et al. 2001). Pretreating tobacco plant with LPS prevented the HR and accelerated the production of SA and subsequent resistance to both host and non-host pathogens (Graham, et al. 1977, Newman, et al. 1997, Newman, et al. 2002); however, symbiotic microorganisms need to avoid this recognition in order to overcome the plant defense for colonization of their host. The LPS from *Sinorhizobium meliloti* is actually able to suppress the elicitor-triggered defense reaction instead of inducing it by blocking ROS production in cell suspension cultures from its symbiotic host *Medicago truncatula* (Albus, et al. 2001) while it induces ROS production in cell suspension cultures from nonhost tobacco; this shows that this suppression is a very specific effect of this LPS for launching a symbiotic relationship (Albus, et al. 2001). Tellstrom et al. (2007) showed that *S. meliloti* LPS can suppress the expression of PR genes triggered by yeast invertase by comparing PR gene expression in two treatments, one with LPS and invertase, and one with invertase alone. The variation in LPS is proposed to be responsible for this difference between suppression and triggering of PTI leading to symbiosis and pathogenicity, respectively (Gohre and Robatzek 2008).

1.1.4. Types of resistance in plants

Resistance in plants is of three different types; Race-specific, race nonspecific and nonhost resistance. Race-specific resistance is qualitative, usually controlled by dominant avirulence or *Avr* genes in the pathogen and controlled by one or a few dominant resistance or *R* genes in a specific plant genotype or cultivar of an otherwise susceptible host species. This resistance is of the "gene-for-gene interaction" type and will be discussed in section 1.3 in detail. Race nonspecific resistance is controlled by many genes and is known as general, quantitative, or partial resistance and is generally durable. This type of resistance depends on pre-existing and induced structural and biochemical defenses provided by dozens or perhaps hundreds of defense-associated genes and the possible ability of pathogenicity-related (PR) proteins to induce pathogens to release molecules that elicit host defenses. Nonhost resistance means the resistance of an entire plant species against a specific pathogen and is the most common and durable form of disease resistance exhibited by plants (Heath 2000). Nonhost resistance is the result of both preformed and inducible defense mechanisms (Heath 2000, Mysore and Ryu 2004), seems to be

under complex genetic control, and can involve multiple defense factors that individually may segregate within host species without compromising overall resistance (Heath 2000). Both nonhost and host resistance sometimes show common components, including deposition of physical barriers, production of reactive oxygen species (ROS), accumulation of antimicrobial compounds, and the HR (Able 2003, Huckelhoven 2007, Zhao, et al. 2005).

It is still not clear whether the signal transduction pathways are similar in race specific and nonhost resistance (Mysore and Ryu 2004). Several signaling components such as ethylene and SA which are important in host resistance to activate the plant defense system also play an important role in nonhost resistance. Transgenic tobacco plants expressing the *Arabidopsis etr1-1* gene responsible for loss of ethylene perception, become susceptible to various nonhost pathogens (Knoester, et al. 1998). Similarly, *sid2* mutant *Arabidopsis* plants, which are defective in salicylic acid synthesis, and *Arabidopsis* plants expressing *NahG* which encodes salicylate dehdroxylase that degrade SA, become susceptible to the cow pea rust fungus *Uromyces vigna* which is a nonhost pathogen for *Arabidopsis* (Mellersh and Heath 2001). Some nonhost resistance genes, such as *NHO1* and *EDS1* in *Arabidopsis,* are involved in nonhost resistance and disruption of theses genes results in the loss of nonhost resistance against nonhost pathogens (Kang, et al. 2003, Parker, et al. 1996), while mutations in *Pen1, Pen2 and Pen3* disrupt the ability of plants to arrest penetration of both host and nonhost pathogens (Collins, et al. 2003, Stein, et al. 2006, Thordal-Christensen 2003).

The nonhost resistance in plants against bacteria, oomycetes, and fungi is of two types; type I and type II (Mysore and Ryu 2004). Type I nonhost resistance is a more basal defense, does not produce an HR or any visible symptoms, and usually arrests the penetration and multiplication of the pathogen in the plant cells (Mysore and Ryu 2004). Type II nonhost resistance is the most common type of nonhost resistance and produces a visible HR at infection sites. Type II nonhost resistance looks phenotypically more similar to a “gene-for-gene based” incompatible interaction which is associated with a HR as a result of recognition of pathogen elicitor molecules. It is hypothesized that type II nonhost resistance may be due to the production of two or more avirulence proteins in the pathogen species which are recognized by all genotypes of a particular plant species and will, therefore, remain a nonhost pathogen (Collins, et al. 2003). For example, a functional type three secretion system (TTSS) is required for bacteria to deliver effectors (which often are avirulence gene products; see next section) into plants and

cause the HR on nonhost plants (Alfano and Collmer 1996). Similarly, INF1, an avirulence factor secreted by *Phytophthora infestans*, is required for producing a HR in nonhost plants such as *Nicotiana benthamiana* (Kamoun, et al. 1998). The type of nonhost resistance produced in nonhost plants depends not only on the pathogen species but also on the plant species. There are several examples in which the same pathogen species produces a type I nonhost resistance on one nonhost plant species and a type II nonhost resistance on another nonhost plant species (reviwed by Klement, et al. 1999, Mysore and Ryu 2004). Similarly, the same plant species can produce a Type I nonhost resistance to one nonhost pathogen and a Type II nonhost resistance to another nonhost pathogen (Lu, et al. 2001, Thomma, et al. 1999).

1.2. Effectors in plant-microbe interactions

Successful pathogens secrete a wide range of so-called "effector" molecules widely believed to function in suppressing host defense responses at multiple levels and/or in evading basal immunity that otherwise would be sufficient to stop infection. Effectors are small molecules and proteins produced by pathogens that can modify host-cells structures or functions, either by contributing to diseases progression (virulence factors and toxins) or induce defense responses (avirulence factors and elicitors) or both (Hogenhout, et al. 2009, Huitema, et al. 2004, Kamoun 2006). Effectors are also thought to have roles in establishing feeding interactions and/or nutrient leakage from the host to the benefit of the pathogens. Phytopathogen effectors are the products of pathogen genes that function inside the plant cells or at the interface of pathogen and host plants (Kamoun 2006, Kamoun 2007). Individual pathogens genomes encode dozens of secreted effectors that are targeted to the host plant apoplast or cytoplasm (Cunnac, et al. 2009, Jiang, et al. 2008, Kamoun 2006, Lindeberg, et al. 2009a, Lindeberg, et al. 2009b, Tyler, et al. 2006).

Effectors from several groups of cellular phytopathogens such as bacteria, oomycetes, fungi and nematodes can enter plant cells (Chisholm, et al. 2006, Huang, et al. 2003, Kamoun 2007). Bacteria use the Type II, Type III and Type IV secretion systems to deliver many effector proteins to the host plant (Cunnac, et al. 2009, Lindeberg, et al. 2006). Oomycetes and fungi likely secrete even more effectors than bacteria; many genomes from phytopathogenic oomycetes and fungi have been sequenced and hundreds of potential effectors have been identified using bioinformatics (Haas, et al. 2009, Jiang, et al. 2008, Kamper, et al. 2006, Tyler,

et al. 2006, Mueller, et al. 2008, Schirawski, et al. 2010). Predicted oomycete effectors, in addition to having an N-terminal signal peptide (SP), carry a host targeting signal (HTS) next to the SP that contains a conserved RXLR and a DEER motif that can target them to the host cell in the absence of the pathogen (Dou, et al. 2008b, Haas, et al. 2009, Jiang, et al. 2008, Kale, et al. 2010, Rehmany, et al. 2005, Tyler, et al. 2006, Whisson, et al. 2007). The predicted sets of effectors from fungi have an N-terminal SP and, in some cases, an RXLR-like motif (Dean, et al. 2005, Kamper, et al. 2006, Kale, et al. 2010, Godfrey, et al. 2010, Schirawski, et al. 2010). Several examples exist where predicted effectors with a SP are shown to have been delivered to the host cytoplasm (Catanzariti, et al. 2006, Gan, et al. 2010a, Kemen, et al. 2005, Khang, et al. 2010). Recently, Kale et al. (2010) showed that at least three effectors; AVRL567 from *Melampsora lini*, AVRLm6 from *Leptosporia maculans*, and AVR2 from *Fusarium oxysporum* f. sp. *lycopersi* (*Fol*) contain a RXLR-like motif and can enter plant cells without the presence of the pathogen. They also showed that the conserved RXLR motif from oomycetes and the RXLR-like motif from other fungi bind specifically to phospholipids, in particular phospahatidylinositol-3-phosphate (PI3P) on the surface of the plasmamembrane and enter the cell through lipid raft-mediated endocytosis (Kale, et al. 2010). Godfrey et al. (2010) recently showed that small secreted protein from haustoria-forming fungal pathogens have Y/F/WXC motif in addition to N-terminal secretion signal. There may be a motif equivalent to RXLR in the effectors from other fungal pathogens that could target them to the host cytoplasm.

1.2.1. Role of effectors in infection

Secreted effectors help the phytopathogens to colonize the host plant at multiple levels. They may facilitate entry into the host, acquisition of nutrients from the host, stop recognition of PAMPs, prevent phytotoxin production, inactivate the plant defense enzymes, or target the PRRs to interfere with the downstream signaling and defense gene expression. Since bacteria have smaller genomes and are normally easier to manipulate, many more plant-pathogenic bacteria have been studied in great detail, as compared to fungal pathogens however, the number of fungal genomes being generated or already available is growing, which is quickly stimulating studies on fungal and oomycete effectors.

1.2.1a. Breaking the physical barrier

After landing on the surface of the host plant, the pathogen either enters the host plant through natural openings (stomata and hydathode) or penetrates the plant surface tissue directly in order to overcome the physical barrier. Bacteria proliferate on the surface of the host plants and use natural openings or wounds to enter the apoplast for colonization. The opening and closing of stomata is highly controlled in plants, by a complex hormone system. Stomata are open during photosynthesis in most plants to allow proper exchange of gasses and phytopathogenic bacteria profit from this opportunity to move towards stomata by sensing compounds released during photosynthesis. The PAMP recognition receptor (PRR) of the guard cells can sense PAMPs such as bacterial flagellin or lipopolysaccharides or lipooligosaccharides from bacteria, which induce the closure of stomata systemically (Gohre and Robatzek 2008). Phytopathogenic bacteria such as *P. syringae* produce coronatine, a phytotoxin that mimics jasmonic acid (JA) to interfere with SA and abscisic acid (ABA) signaling for reopening stomata so that pathogenic bacteria can gain access to the host apoplast (Melotto, et al. 2006). It is not yet known whether MAMP associated defense pathways also close stomata to eukaryotic pathogens and whether these pathogens use effectors in a similar way to overcome this hurdle. After getting into the plant apoplast, the next physical barrier to the phytopathogen is the plant cell wall which prevents them from obtaining nutrients. To promote nutrient leakage from the cytosol into the apoplast, bacteria use a Type II secretion system to secret lytic enzymes that degrade the cell wall locally. In the generated open channel, a component of the Type III secretion system, a nano-scale injection structure, is then formed which penetrates the cell wall and cell membrane (Gohre and Robatzek 2008). At the same time, these bacteria secrete effector molecules to suppress the host defense that is activated by danger associated molecular patterns (DAMPs) from the degrading cell wall molecules (Jha, et al. 2007). Powdery mildew fungi, such as *B. graminis* f. sp. *hordei* penetrate the cuticle of plant cells directly through a special penetration structure, called the appressorium. It has been shown that *B. graminis* f. sp. *hordei* secreted effectors AVRa10 and AVRk1 increase the penetration efficiency on susceptible barley cultivars but the exact mechanism is not yet known (Ridout, et al. 2006).

1.2.1b. Disarming plant defense enyzymes

Plants produce antimicrobial enzymes like proteases, hydrolases, glucanases, and chitinases that can degrade the cell wall of invading pathogenic fungi in the apoplast without detrimental effects to the plant (Lucas 1998). This has a dual role, the degradation of cell walls can attenuate fungal growth on the one hand, while on the other hand, the molecules released from degraded cell walls serve as elicitors for inducing plant defense. Pathogens use effector molecules either to stop the delivery of these antimicrobial enzymes and compounds by preventing their secretion or by inhibiting their activity after they are secreted (Bent and Mackey 2007). The apoplastic fungus *Cladosporium fulvum* secretes AVR2, a cysteine protease inhibitor, during infection that binds directly to RCR3, a tomato cysteine protease, to protect the fungus from the deleterious effect of the enzyme. AVR2 also promotes virulence for other fungal pathogens that cause disease in tomato, such as *Verticillium dahliae* and *Botrytis cinerea*, when expressed heterologously in *Arabidopsis* (van Esse, et al. 2008). Similarly, effector AVR4, a chitin binding lectin from *C. fulvum*, binds to chitin of fungal cell walls to protect it from chitinases of the host plant, tomato (van den Burg, et al. 2006). AVR4 can also protect chitin against plant chitinases in the cell wall of other fungi, such as *Trichoderma viride* and *Fusarium solani* f. sp. *phaseoli* (van den Burg, et al. 2006). In this way, AVR4 not only protects the fungi from hydrolysis by plant chitinases but also keeps chitin fragments from eliciting PTI (Libault, et al. 2007). ECP6 from *C. fulvum* has a Lys-M domain that binds to carbohydrates including chitin and protects the pathogen from plant chitinases or may be involved in scavenging of chitin fragments that are released during cell wall degradation by plant chitinases, thus, preventing them from inducing PTI (Bolton, et al. 2008).

The oomycete pathogen, *P. infestans*, is known to secrete a suite of Cysteine and Kazal family protease inhibitors (Tian and Kamoun 2005, Tian, et al. 2007, van Esse, et al. 2008). The tomato papain-like protease, PIPI, which is induced by SA, is blocked by the EPIC2B inhibitor of *P. infestans* (Tian and Kamoun 2005, Tian, et al. 2007, van Esse, et al. 2008). PIP1 is related to RCR3, and a new report shows that AVR2 from *C. fulvum* can inhibit PIP1 and two other cystein protease, aleurain and TDI65, in plants (Rooney, et al. 2005, Shabab, et al. 2008, van Esse, et al. 2008). Also EPIC1 and EPIC2B from *P. infestans* can bind and inhibit RCR3, similar to AVR2, but unlike AVR2, these EPICs do not elicit an HR on Cf-2/Rcr3^{pimp} tomato plants suggesting that *P. infestans* evolved stealthy effectors that can inhibit tomato proteases without

activating defense responses (Song, et al. 2009). These findings show that effectors from different pathogens can target the same apoplastic enzymes to increase pathogen fitness in the host (Shabab, et al. 2008). Other effectors, like EPI1 and EPI10 from *P. infestans*, target the subtilisin-like serine protease of tomato P69B, a PR protein (Tian, et al. 2004, Tian and Kamoun 2005, Tian, et al. 2007). AVRP123, a secreted protein from *M. lini*, the flax rust fungus, also shows similarity to Kazal serine protease inhibitors (Catanzariti, et al. 2006). The soybean pathogen, *Phytophthora sojae*, secretes glucanase inhibitor proteins, GIP1 and GIP2, that target the endo-β-1,3-glucanase-A of the host plant to protect the pathogen during infection and also to prevent PTI induced by oligoglucoside (Rose, et al. 2002). Bacteria also secrete plant cell wall-degrading enzymes locally in order to construct the Type III secretion system and use effectors such as HOPP1 to suppress defense induced by DAMPs in the *N. benthamiana* apoplast (Gust, et al. 2007, Oh, et al. 2007). HOPP1 either sequesters or processes the fragments that are produced during cell wall degradation so that they cannot function as PAMPs (Gohre and Robatzek 2008). These examples of disarming the apoplastic enzymes from plants by different classes of pathogen (fungi, oomycets and bacteria) show the importance of effectors in preventing PTI and promoting pathogen fitness in the host.

1.2.1c. Suppression of receptor activation

The recognition of PAMPs by PRRs of plants leads to the activation of defense responses against the pathogen by triggering a cascade of defense signaling. It was proposed in the beginning that resistance induced by PRRs is only basic and not as strong as that induced by resistance genes, but it is clear now, at least in the case of FLS2 (the flagellin receptor), that the contribution of PRRs towards overall resistance is huge (de Torres, et al. 2006, Hann and Rathjen 2007, He, et al. 2006). PRRs make significant contributions to resistance in nonhost pathogens. Bacterial strains mutated in the Type III secretion system show that suppression of PTI by effectors is important for full virulence in these pathogens (Hauck, et al. 2003, Kim, et al. 2005). Several effectors, particularly the Type III secretion system effectors of phytopathogenic bacteria, target these PRRs directly to jam all the downstream resistance responses (Blocker, et al. 2008, Jamir, et al. 2004, Jones and Dangl 2006). AVRPTO and AVRPTOB are two unrelated Type III effectors of *P. syringae* that inhibit FLS2 recognition, upstream of MAPKKK signaling in the plant (He, et al. 2006). Several PTI responses such as the production of ROS, MAPK cascade

induction, elicitation of PR genes, and callose accumulation are prevented by the action of AVRPTO when it is localized in the membrane (He, et al. 2006). The AVRPTO and AVRPTOB target the resistance gene product, PTO, in tomato, a protein which is guarded by resistance gene product PRF and induces an HR (Kim, et al. 2002). The *Arabidopsis* genome did not contain an ortholog of PTO but the kinase domains of FLS2, BAK1, and EFR show significant homology to PTO (Chinchilla, et al. 2007, Kim, et al. 2002). It has been shown by structural modeling that the kinase domain of PTO is similar to FLS2 and EFR (Xiang, et al. 2008). AVRPTO interacts with FLS2 and EFR both *in vitro* and in protoplast, which shows that it targets multiple PRRs and most likely the same, may be true for AVRPTOB (Xiang, et al. 2008). The C-terminus of AVRPTOB carries a functionally active E3 ligase activity that ubiquitinates FEN kinase, a member of the PTO family, for degradation; that activity can then prevent an HR in tomato plants lacking PTO (Janjusevic, et al. 2006, Rosebrock, et al. 2007). AVRPTO and AVRPTOB can also suppress nonhost HR in *N. benthamiana* induced by FLG22 or INF1 of *P. infestans* (Hann and Rathjen 2007). DSPA/E that is related to the AVRE family of the apple fire blight pathogen, *Erwinia amylovora*, interacts directly with four apple proteins that are putative receptor-like kinases (RLKs; (Meng, et al. 2006). Several oomycete RXLR effectors can suppress host cell immunity in a similar manner, as do the bacterial Type III secretion system effectors. AVR3A from *P. infestans* can block an HR induced by INF1 (Bos, et al. 2009). Similarly, AVR1B from *P. sojae* also suppresses programmed cell death triggered by the mouse protein, BAX, in plants and yeast (Dou, et al. 2008a). Several alleles of ATR1 and ATR13 from *Hyaloperonospora arabidopsidis* increased the virulence of *P. syringe* DC3000 on susceptible *Arabidopsis thaliana* when these effectors were delivered by the *P. syringae* Type III secretion system (Sohn, et al. 2007). ATR13 targets PTI by suppressing callose accumulation and ROS production in susceptible *A. thaliana* (Sohn, et al. 2007).

1.2.1d. Suppression of *R* gene-triggered resistance

Phytopathogenic fungi like *Fusarium oxysporum* f.sp. *lycopersici* can avoid host defense by evolving effectors that can suppress *R* gene-triggered resistance (Houterman, et al. 2008). AVR3 and AVR2 from *F. oxysporum* f.sp. *lycopersici* are required for full virulence on tomato plants but they are also recognized by tomato lines that have resistance genes *I-3* or *I-2*, respectively, that trigger an HR and cause arrest of the pathogen (Huang and Lindhout 1997, Rep, et al. 2005,

Rep, et al. 2004). In contrast, AVR1 is a small cysteine-rich secreted protein from *F. oxysporum* f.sp. *lycopersici* that is recognized by resistance gene *I* or *I-1* but is not required for virulence. Tellingly, AVR3 is present in all *F. oxysporum* f.sp. *lycopersici* strains analysed, while Avr1 is present only in *F. oxysporum* f.sp. *lycopersici* strains that are virulent on I-3 lines. Houterman et al. (2008) showed that AVR1 actually suppresses the resistance triggered by I-2 and I-3, as the transformation of *Avr1* to *F. oxysporum* f.sp. *lycopersici* strains that were avirulent on *I-2* or *I-3* became virulent on these lines. It is proposed that *F. oxysporum* f.sp. *lycopersici l* strains acquired *Avr1* as a mechanism of partial functional redundancy, so that they can avoid the consequences of losing *Avr3* and probably *Avr2* that are required for full virulence (Stergiopoulos and de Wit 2009). In the flax rust fungus, *M. lini*, the interaction of *AvrL567* in strain CH5-89 with the flax cultivar Barnes that contains the *L7* gene is inhibited by an inhibitor gene and thus results in a lower virulence reaction (Lawrence, et al. 2010).

1.2.1e. Down-regulation of defense signaling

As already mentioned, the perception of a PAMP by a PRR triggers a cascade of defense signaling including MAPK cascades. In *Arabidopsis*, the recognition of FLG22 by FLS2 induces downstream signaling through a MAPK cascade (Asai, et al. 2002, Gomez-Gomez and Boller 2000). Bacterial Type III secretion system effectors use different mechanisms to dephosphorylate the MAP kinase signaling components in order to suppress the defense response (Gohre and Robatzek 2008). HOPAI1 encodes an enzyme, phosphothreonine lyase, that removes a phosphate group from phosphothreonine residues of MAPKs to stop phosphorylation, which in turn blocks FLG22 signaling at early stages (Zhang, et al. 2007). Protein pull-down assay showed that HOPAI1 directly interacts with both MAP3 and MAP6 kinases (Zhang, et al. 2007). The inactivation of MAPK by HOPAI1 also stops downstream defense responses, such as the production of ROS and the expression of *PR* genes (Zhang, et al. 2007). HOPAO1, another Type III secretion system effector, has a protein tyrosine phosphatase motif and posseses this enzymatic activity *in vitro* (Bretz, et al. 2003, Espinosa, et al. 2003). The transient expression of HOPAO1 in *N. tabacum* suppresses an HR induced by the constitutively active MAPKK, NtMEK2, in *N. tabacum* (Espinosa, et al. 2003). The heterologous expression of HOPAO1 in *Arabidopsis* also suppresses PAMP-induces ROS production, callose deposition, and thereby enhances virulence and multiplication of *P. syringae* pv tomato (*Pst)* DC3000 *hrpA* mutants

(Underwood, et al. 2007). In that study, transgenic *Arabidopsis* plants expressing a catalytically inactive derivative did not show these phenotypes, which argues that the phosphatase activity is required for HOPAO1 function (Underwood, et al. 2007).

1.2.1f. Alteration of the plant defense transcriptome

The perception of PAMPs such as flagellin changes the expression of at least 1,000 genes in *Arabidopsis* (Zipfel, et al. 2004). As discussed, bacterial Type III secretion system effectors obstruct the defense signaling on the one hand, while on the other hand, they target the plant transcriptome directly. This can be achieved by different mechanisms. Several effectors change RNA stability. For example, HOPU1, a mono-ADP-ribosyltransferase, acts on glycine-rich RNA-binding proteins such as AtGRP7 and AtGRP8 which are RNA chaperones (Fu, et al. 2007); thus, HOPUI changes the plant transcriptome by reducing transcripts and the expression of defense response genes. *Arabidopsis grp7* mutants promote growth of *P. syringae* pv tomato DC3000 compared to the wild type (Fu, et al. 2007). Besides HOPUI, HOPO1-1 and HOPO1-2 also encode ADP-RTs.

Some pathogen effectors act as transcription factors and can induce the expression of host genes for their own benefits. For example, the AVRBS3 family of effectors from *X. campestris* pv *vesicatoria* has plant nuclear localization signals and binds to an "upa-box" (upregulated by AVRBS3) that is found in the promoter of *Upa20*, a master regulator of cell size inducing hypertrophy, and several other host genes, ensuring proper nutrient supply for pathogen multiplication (Gurlebeck, et al. 2006, Kay, et al. 2007, Szurek, et al. 2002); however, in resistant plant cultivars, the promoter of *Bs3* also carries an "upa-box" and, thus, binding of the ABRBS3 effecor induced transcription of *Bs3* and cell death (Kay, et al. 2007). This shows that under selection pressure, plants can evolve to recognize effectors and use them for their own defense.

1.2.1g. Destruction of antimicrobial compounds

Microbial effectors also attack and degrade antimicrobial compounds and proteins that play roles in PAMP perception, defense signaling, or any other defense reaction. This degradation is achieved either by protease action on the plant substrate or by exploiting the plant protein degradation machinery (Zeng, et al. 2006). SUMO (small ubiquitin-like modifier) modification

or sumoylation controls several processes in plants like pathogen infection, abiotic stress, hormone signaling, and flowering time (Hanania, et al. 1999, Kurepa, et al. 2003, Lois, et al. 2003, Murtas, et al. 2003). It was recently discovered that several bacterial Type III secretion system effectors such as YOPJ, XOPD, YOPT, AVRXV4, AVRPPHB and AVRRPT2 possesses cysteine protease functions, suggesting that the degradation of host proteins is an important approach to subvert plant defenses (Hotson and Mudgett 2004). YOPJ and XOPD effectors have deSUMOylating activities and XOPD accumulates in the subnuclear foci, revealing its function in deSUMOylation of transcription factors (Hotson, et al. 2003, Hotson and Mudgett 2004). The YOPT protease has a cytotoxic effect that destroys the actin cytoskeleton during the entry into the cell, resulting in severe cell damage (Shao and Dixon 2003). AVRPPHB effector cleaves itself before entering into host plant by the TTSS and is fatty acylated to target PBS1 cleavage (Shao, et al. 2002). In resistant plants, the cleavage and PBS1 kinase activities are required for RPS5 resistance (Shao, et al. 2002). It has also been suggested that AVRRPT2 functions as a cysteine protease *in planta* (Hotson and Mudgett 2004). In *Arabidopsis* plants, AVRRPT2 degrades RIN4 which in turn activates RPS2 to develop resistance to the pathogen (Mudgett and Staskawicz 1999). Besides AVRRPT2, two other *P. syringae* effectors, AVRRPM1 and AVRB, independently target RIN4 and induce its phosphorylation, which in turn induces RPM1 resistance (Mackey, et al. 2002). As AVRRPT2 degrades RIN4, AVRRPM1 and AVRB cannot activate RPM1 in the presence of AVRRPT2 (Axtell and Staskawicz 2003, Mackey, et al. 2003). It is proposed that RIN4 might be acting as an adapter for holding multiple PRR signaling pathways under negative control (Gohre and Robatzek 2008).

TTSS effectors also exploit the proteasome degradation machinery of host plants for degrading host defense related proteins (Angot, et al. 2007). AVRPTOB is a modular protein that contains an N-terminal domain that induces the HR by interacting with PTO/PRF in a gene-for-gene manner (Abramovitch, et al. 2003, Jamir, et al. 2004). The C-terminal domain of AVRPTOB is capable of suppressing the HR triggered by AVRPTO/PTO recognition in *N. benthamiana* and the HR induced by other bacterial Type III secretion system effectors, fungal specific HR inducing proteins, and even pre-apoptotic mouse BAX protein (Abramovitch, et al. 2003, Jamir, et al. 2004). C-terminal domain of AVRPTOB carries an E3-ligase activity and is capable of auto-ubiquitination and possibly of ubiquitination of plant substrates (Abramovitch, et al. 2006, Abramovitch, et al. 2003, Janjusevic, et al. 2006). MAMPs from several bacterial and

fungal phytopathogens induce cell wall-based responses. Papilla formation, which is a localized cell wall thickening mainly by addition of callose near pathogen penetration sites, is induced by a variety of phytopathogens (reviewed by Bent and Mackey 2007). During papilla production, cellular vesicles deliver cell wall reinforcement and antimicrobial compound to the site of pathogen penetration (Robatzek, et al. 2006). Several effectors can act on proteins involved in this transportation and redirect their cargo to suppress MAMP-induced callose deposition. HOPM1, a Type III secretion system effector from *P. syringae*, is required for virulence and able to suppress host cell wall-associated defense (DebRoy, et al. 2004). HOPM1 manipulates the plant ubiquitination system to alter vesicle trafficking. It interacts specifically with AtMIN7, one of the *Arabidopsis* ARF-GEFs (adenosine diphosphate ribosylation factor guanine nucleotide exchange factor) that is involved in vesicle trafficking (Nomura, et al. 2006). The interaction of HOPM1 with AtMIN7 mediates proteasome-based degradation of AtMIN7 and, thus, callose deposition is prevented (Nomura, et al. 2006). HOPM1 by itself did not have any classical E3-ubiquitin ligase features, leading to the hypothesis that HOPM1 may act as an adapter protein mediating the detection of AtMIN7 by the plant ubiquitin/26S proteasome system (Angot, et al. 2007). Similarly, AVRPTO also suppresses cell wall-based defenses independent of SA signaling (Hauck, et al. 2003). It is possible that AVRPTO interferes with vesicle trafficking in a similar manner as HOPM1, as a yeast two-hybrid assay showed that it interacts with two Rab-GTPases (Hauck, et al. 2003).

1.2.1h. Killing of host cells

Necrotrophic phytopathogens produce several phytotoxins in addition to cell wall hydrolyzing enzymes in order to kill the host tissue for colonization during infection. Some necrotrophic fungi produce proteinaceous effectors, also called host selective toxins (HST), that are required for infection on susceptible host plants that have the corresponding dominant receptor gene (Wolpert, et al. 2002). This represents a situation opposite of the classical gene-for-gene interaction in which a dominant gene is required for disease resistance rather than pathogenicity (Wolpert, et al. 2002). Two wheat necrotrophs, *Stagonospora nodorum* and *Pyrenopora tritici-repentis*, produce several host-specific peptide effectors, such as PTRTOXA, SNTOX1, SNTOX2, and SNTOX4, that are recognized by their corresponding dominant susceptibility genes in wheat (*TsN1, Snn1, Snn2* and *Snn4*) to causes disease (Abeysekara, et al. 2009, Friesen,

et al. 2007, Liu, et al. 2006, Liu, et al. 2004, de Wit, et al. 2009, Manning, et al. 2009). PTRTOXA, the best-studied effector from *P. tritici-repentis* has an N-terminal secretion signal, followed by an RGD domain for host targeting and a C-terminal domain with effector function (Manning, et al. 2007, Sarma, et al. 2005). After entering the host cell, PTRTOXA was reported to enter the chloroplast and interfere with TOXABP1 function of the chloroplast, thereby affecting photosystem I and II function in a light-dependent manner (Manning, et al. 2007). PTRTOXA is an ortholog to SNTOX1 from *S. nodurum* and both effectors are recognized by the same wheat susceptibility gene, *Tsn1* (Liu, et al. 2006).

Some phytopathogenic bacteria, oomycetes, and fungi produce NEP1-like proteins (NLPs) that are toxic to only dicotyledonous plants, possibly by the different molecular compositions of dicot and monocot cell membranes (Gijzen and Nurnberger 2006, Ottmann, et al. 2009, Pemberton and Salmond 2004). The common heptapeptide motif, GHRHDWE, and two conserved cysteine residues make it structurally similar to actinoporins, cytolytic toxins from marine organisms (Gijzen and Nurnberger 2006, Ottmann, et al. 2009). Hemibiotrophs, such as *P. infestans* and *P. sojae*, produce NLPs such as NPP1 and PiNPP1, in the late necrotrophic phase which could contribute to disease development with their cytolytic activities (Kanneganti, et al. 2006, Qutob, et al. 2002).

1.2.1i. Suppression of host defense by symbionts

Symbiotic microorganisms also secrete effectors into host plants to suppress host defense responses, a condition essential for their lifestyle. Many rhizobial strains use the Type III secretion system to deliver effectors into host cells in a similar way that pathogenic bacteria suppress host defense responses (Deakin and Broughton 2009). The relationship between symbiotic bacteria and their host plants is very specific, which is determined by molecular signals recognized by the two organisms. For example, each rhizobial strain can establish symbiosis with only limited species of host plants (Perret, et al. 2000, Yang, et al. 2010). The host range is defined by bacterial recognition of flavonoid compounds secreted by the host, which induce the NOD factors and the perception of these NOD factors by the host plants receptors (Geurts, et al. 1997, Limpens, et al. 2003, Radutoiu, et al. 2003, Radutoiu, et al. 2007). The transformation of *NFR1* and *NFR5* Nod-factor receptors from *Lotus japanicus* into *M. truncatula* make it a host for *Mesorhizobium loti* that usually has a symbiotic relationship only

with *L. japanicus* (Radutoiu, et al. 2003, Radutoiu, et al. 2007). LPSs from phytopathogenic bacteria are recognized by plants as elicitors of PTI, while symbiotic bacteria such as rhizobia use these LPSs to suppress host defenses and assist in making infection threads and inducing nodule growth (D'Haeze and Holsters 2004, Jones, et al. 2008). It was reported a few decades ago that dominant *R* genes present in plants regulate symbiotic relationships with particular rhizobial strains which is similar to a gene-for-gene interaction in plant-pathogen interaction (Caldwell 1966, Devine and Kuykendall 1996). Two *R* genes, *Rj2* and *Rfg1* have been cloned from soybean, which restrict symbiotic relationships between *Bradyrhizbium japanicum* and *Sinorhizobium fredii* in a gene-for-gene manner (Yang, et al. 2010). In the fungal kingdom, the genome of *Laccaria bicolor*, an ectomycorrhizal fungus, revealed more than 3,000 predicted secreted proteins of which 10% are small secreted proteins (SSPs) of the effector-type (Martin, et al. 2008, Martin and Selosse 2008). Some of these SSPs are homologous to rust fungus "haustoria-expressed secreted proteins" (HESPs) and are differentially expressed during infection, suggesting a possible role during infection and evading the host defense (de Wit, et al. 2009).

1.3. Gene-for-gene or *R* and *Avr* interaction

Adapted pathogens secrete effector molecules into the host plant to surmount physical barriers, neutralize preformed antimicrobial compounds, and overcome PTI. As a result, during co-evolution of plant host and adapted pathogen, plants have developed very sophisticated recognition systems, usually encoded by *R* genes to recognize these effectors or their action and trigger defense responses; this induced resistance has recently been called effector-triggered immunity (ETI) and leads to a rapid and enhanced defense response in the host plant often including HR (Shirasu and Schulze-Lefert 2000). These effectors are called avirulence (Avr) factors since they activate the plant defense system and make the pathogen unable to cause disease when the plant has the corresponding *R* gene (Jones and Dangl 2006). This genetically superimposed *R* and *Avr* interaction is called "gene-for-gene" resistance, or host/cultivar-level resistance, as particular cultivars of the host with a certain *R* gene product recognize an *Avr* gene product from a particular race of the pathogen. When a pathogen cannot cause disease in plants, the interaction is incompatible, the pathogen is avirulent, and the plant is resistant. The

hypothesis of "gene-for-gene" resistance, first proposed by Flor (1942), states that for every dominant *Avr* gene in the pathogen, there is a corresponding dominant *R* gene in the resistant host. The (genetic) interaction between these gene products leads to activation of defense responses in the host and suppresses pathogen growth (Flor 1942). Some *Avr* genes encode effectors molecules that suppress host defense and, thus, act as virulence factors when the plant does not have the corresponding resistance gene. ETI represents the qualitative, secondary layer of resistance and leads to an evolutionary arms race between the pathogen and the plant in which the pathogen either mutates or loses the effectors to avoid recognition by the host or develops new effectors to suppress ETI, while the plant develops new *R* genes to recognize the mutants or new effectors (de Wit 2007, Bent and Mackey 2007, Jones and Dangl 2006).

The cloning and characterization of various *Avr* and *R* genes in the past three decades has increased our knowledge of the biochemical and molecular basis of the gene-for-gene interaction (Hammond-Kosack and Parker 2003). The first *Avr* gene was cloned from a bacterium in 1984 (Staskawicz, et al. 1984), which was followed by the cloning and characterization of more than 40 bacterial *Avr* genes in the following decades (Mudgett 2005, Van't Slot and Knogge 2002). The cloning and characterization of *Av*r genes from fungi and oomycetes lagged behind because of these organisms' larger genome sizes and sometimes inefficient transformation systems. The first *Avr* gene from a fungus was isolated from C. *fulvum* in 1991 (van Kan, et al. 1991) and the first oomycete *Avr* gene was cloned from *P. sojae* and *P. infestans* in 2004 (Shan, et al. 2004). Fungal *Avr* genes are mainly isolated from ascomycetes, such as *C. fulvum, Rynchosporium secalis, B. graminis, Magnaporthe oryzae, F. oxysporum,* and *L. maculans*, but a handful have been reported from a basidiomycete, the flax rust fungus, *M. lini* (Catanzariti, et al. 2006). Several more oomycete *Avr* genes have since been isolated from *H. arabidopsidis, P. sojae*, and *P. infestans* (Table 1.1). The sequencing of the complete genomes of many bacterial, fungal, and oomycete plant pathogens has revealed a myriad of effectors, many of which are potential avirulence genes that can trigger ETI.

Avr genes isolated and characterized to date differ among one another both in sequence and function, and those from different plant pathogens do not seem to share many common features (Agrios 2005). More than 40 bacterial *Avr* genes have been cloned and sequenced primarily from *Pseudomonas* and *Xanthomonas* species and most of them share little or no homology to each other with few exceptions, such as in the *avrbBs3* and *avrRxv/yopJ* families

(Deslandes, et al. 2003, Lahaye and Bonas 2001). The AVRBS3-like proteins from all *Xanthomonas* share 90-97% sequence identity in a central region of nearly identical 34-amino acid repeats (Lahaye and Bonas 2001). Besides the repeat domain, all members of the AVRBS3 family contain nuclear localization signals (NLS) and an acidic transcriptional activation domain. Bioinformatic analyses showed that all family members of AVRRXV/YOPJ have common invariant residues in a putative protease catalytic site (Orth, et al. 2000). All of the fungal and oomycete *Avr* genes characterized to date encode small proteins (28-311 amino acids), except the ACE1 of *M. grisea* which has 4,034 amino acids, and all have a secretion signal/protein transport motif at the N-termini (Ellis, et al. 2006); however, it has been shows that AVRa10 and AVRk1 from *B. graminis* f. sp. *hordei* do not have secretion signals like other fungal AVR proteins (Ridout, et al. 2006). In spite of the absence of secretory signals, these AVR proteins are recognized by intracellular barley resistance proteins when expressed transiently *in planta.* It has been suggested that AVRa10 and AVRk1 may be secreted from the fungus by non-endomembranous pathways (Ridout, et al. 2006).

1.3.1 Fungal *Avr* genes

Several *Avr* genes have been cloned and characterized from extracellular fungi such as *C. fulvum, F. oxysporum* f. sp. *lycopersici, R. secalis, M. oryzae* and *L. maculans* and from obligate biotrophic fungal pathogens that produce haustorial feeding structures within cells, such as *B. graminis* and *M. lini* (Table 1.1).

1.3.1a. *Cladosporium fulvum*

C. fulvum is an apoplastic fungal pathogen of the ascomycete subgroup that reproduces asexually and causes leaf mold of tomato (de Wit, et al. 1997, Joosten and de Wit 1999, Thomma, et al. 2005). Four avirulence genes have been cloned: *Avr2, Avr4, Avr4E* and *Avr9*, which all encode small secreted cystein-rich effector proteins that are recognized in tomato by CF2, CF4, CF4E and CF9 resistance proteins, respectively, that mediate the HR in tomato (de Wit, et al. 1997, Joosten and de Wit 1999, Thomma, et al. 2005). The virulence function of AVR2 and AVR4 has been discussed previously in the effectors section. AVR4E is a secreted cysteine-rich, 101 amino acid (aa) protein that induces HR in tomato lines that contain the *Cf-4E* gene; it does not have a known virulence function (Westerink, et al. 2004). Several field isolates that overcome CF-4E-

mediated resistance reveal point mutations in AVR4E or a complete loss of the *Avr4E* gene, which shows that it probably does not affect fitness of the pathogen (Stergiopoulos, et al. 2007). *Avr9* encodes a 28 aa mature protein with six cycteine residues after it is processed by plant and fungal proteases at its C- and N-termini (Ackerveken, et al. 1993, van Kan, et al. 1991). Structurally, AVR9 is similar to carboxypeptidase inhibitor but no definitive function has been identified so far (Van den Ackerveken, et al. 1993, van den Hooven, et al. 2001, van Kan, et al. 1991). All natural strains of *C. fulvum* that overcome CF9 resistance lack *Avr9*, suggesting that it is not required for full virulence (Stergiopoulos, et al. 2007). *C. fulvum* deleted for *Avr9* is fully virulent on tomato plants, but the heterologous expression of *Avr9* in tomato plants makes it more susceptible to *C. fulvum* strains lacking *Avr9*, indicating some (redundant) virulence function (de Wit, et al. 2009, Marmeisse, et al. 1993).

Besides Avr effectors, four extracellular cysteine-rich proteins (ECP), such as ECP1, ECP2, ECP4 and ECP5, have been cloned and characterized from *C. fulvum* that induce the HR in tomato lines containing the corresponding *Cf-Ecp* resistance genes (de Kock, et al. 2005, Lauge, et al. 2000). Bolton et al. (2008) reported the cloning of *Ecp6* and *Ecp7* from *C. fulvum* but the corresponding tomato lines that recognize these genes have not yet been identified. ECPs are present in all strains of *C. fulvum* and are secreted during infection. They contain an even number of cysteine residues that are most likely involved in disulphide bridge formation to protect them from apoplastic proteases (Luderer, et al. 2002a). Three of the ECPS, ECP1, ECP2 and ECP6, have virulence functions on the host plants, based on data showing that deletion or suppression of expression of these genes reduced virulence of *C. fulvum* on host plants (Bolton, et al. 2008, Lauge, et al. 1997). Orthologs for AVR4 and ECP6 have been identified in several fungal species because of the presence of CMB14 and LysM domains in these proteins (Bolton, et al. 2008). The ortholog of AVR4 and ECP2 have been identified in *Mycosphaerella fijiensis* that causes black Sigatoka disease of banana (Stergiopoulos et al. 2010). The *M. fijiensis* ortholog of AVR4 induces HR in tomato lines containing the corresponding *Cf4* gene and binds to chitin of fungal cell walls to protect against cell wall degradation, similar to *C. fulvum* AVR4 (Stergiopoulos, et al. 2010). Similarly, ECP2 of *M. fijiensis* is a functional ortholog of *C. fulvum* CF-ECP2 and is recognize by CF2 of tomato to induce a HR, while in the absence of CF2, it promotes virulence on tomato plants (Stergiopoulos, et al. 2010).

1.3.1b. *Rynchosporium secalis*

The imperfect fungus, *R. secalis*, causes leaf scald disease on barley by secreting low molecular-weight toxic proteins. Three of these effectors, designated as NIP1, NIP2 and NIP3, have been cloned (Hahn, et al. 1993, Rohe, et al. 1995, Steiner-Lange, et al. 2003) and encode small secreted toxic proteins in a genotype non-specific manner on barley and related cereal plant species. Mature NIP1 is a 60 aa protein with ten cysteine residues that are involved in intramolecular disulphide bond formation. NIP1 triggers specific defense responses without a HR on barley cultivars that have the corresponding resistance gene, *Rrs1* (Lehnackers and Knogge 1990). The injection of NIP1 into leaves of barley and other cereal plant species causes scald-like lesion formation (van 't Slot, et al. 2007, Wevelsiep, et al. 1991). A *nip1* disruption mutant of *R. secalis* is slightly less virulent than the wild type on susceptible plants, demonstrating its role in virulence (Knogge and Marie 1997). *R. secalis* virulent strains overcome *Rrs1* resistance of barley either by a point mutation in the ORF that results in a single aa substitution or by jettison of the *Nip1* gene (Rohe, et al. 1995). It has been shown that NIP1 interacts with a single plasma membrane receptor (different from RRS1) that is involved both in necrosis and defense induction (van 't Slot, et al. 2007). A field population study of this pathogen showed a positive diversifying selection on the *Nip1* locus, as three out of the 14 isoforms gained virulence on *Rrs1* barley lines and a high deletion frequency was observed in the *Nip1* locus compared to *Nip2* and *Nip3* (de Wit, et al. 2009). The deletion frequency of *Nip1* was higher than the occurrence of the point mutation that gains virulence indicating a reduced fitness penalty for the loss of the *Nip1* gene. *Nip2* encodes a 109 aa protein with a predicted secretion signal of 16 aa and a mature protein with six cysteine residues, while *Nip3* encodes a 115 aa protein with a predicted secretion signal of 17 aa and a mature protein with eight cysteine residues (de Wit, et al. 2009).

1.3.1c. *Blumeria graminis*

Powdery mildews are biotrophic ascomycete fungi that cause diseases on various mono- and dicotyledonous plant species, including food crops, feed crops and ornamental plants (Bushnell 2002). They are obligate biotrophs that need a living host for growth and reproduction and produce intracellular feeding structures, the haustoria, in the epidermis of their host plants

(Glawe 2008, Yarwood 1957). *B. graminis* f. sp. *hordei* causes powdery mildew on barley and is the most thoroughly studied powdery mildew fungus. It interacts with its host in a "gene-for-gene" manner (Both, et al. 2005, Zhang, et al. 2005). The gene-for-gene interaction revealed that there are more than 85 dominant or semi-dominant mildew (*Ml*) resistant genes that recognize different races of *B. graminis* f. sp. *hordei*, including 28 highly similar genes at the *Mla* locus on barley chromosome 5 (Jensen, et al. 1980). Seven *Mla* genes at this locus have been cloned and they all encode closely related intracellular coiled-coil nucleotide-binding site, leucine-rich repeat (CC-NBS-LRR) type R proteins (Halterman, et al. 2001, Shen, et al. 2003) that recognize different *B. graminis* f. sp. *hordei* avirulence proteins (Halterman, et al. 2001, Halterman and Wise 2004). Two effectors proteins, AVRa10 and AVRk1, have been isolated from *B. graminis* f. sp. *hordei*; they are virulence factors in that they promote infection on susceptible barley cultivars (Ridout, et al. 2006); however, these effectors are recognized by the barley resistance genes, *Mla10* and *Mlk1*, respectively, where they induce a HR (Ridout, et al. 2006). Both *Avr* genes belong to multi-gene families that have more than 30 paralogs and they also have orthologs in other *forma speciales* that are pathogenic on other grasses (Ridout, et al. 2006). The AVRa10 and AVRk1 predicted proteins lack any N-terminal secretion signal or host targeting sequence, but it has been shown by fluorescence microscopy that the MLA10 protein is present intracellularly, both in the cytoplasm and the nucleus of invaded barley cells. This means that AVRa10 is taken up by the cell by an as yet unknown mechanism (Bieri, et al. 2004, Shen, et al. 2007). Besides AVRa10 and Avrk1, two other *Avr* genes, *Avra22* and *Avra12*, have been mapped recently (Skamnioti, et al. 2008).

1.3.1d. *Melampsora lini*

M. lini is an obligate biotrophic fungus that belongs to the basidiomycetes and causes flax rust disease not only in flax but also in other species of the genus *Linum* (Stergiopoulos and de Wit 2009). A number of flax R proteins have been analyzed; these are highly polymorphic cytoplasmic TIR-NBS-LRR proteins that recognize effector proteins that are delivered into flax cells during colonization. This interaction triggers HR and arrests growth of the fungi in a gene-for-gene manner (Lawrence, et al. 2007). The genetic analysis of *M. lini* with its host plant flax revealed at least 30 *Avr* genes and corresponding *R* genes (Ellis, et al. 1997). Several *Avr* genes have been cloned from *M. lini*, mainly from four different loci: *AvrL567*, *AvrM, AvrP123* and

AvrP4. These genes encode haustoria expressed secreted proteins (HESPs), suggesting that they have virulence functions, but elicit defence responses in hosts that have the corresponding *R* genes (Catanzariti, et al. 2006). The *AvrL567* locus has a cluster of three polymorphic *Avr* genes: *AVRL567A*, *AVRL567B* and *AVRL567C*. All three encode 127 aa mature proteins after cleavage of a 23 aa signal peptide and are recognized directly by the L5, L6 and L7 proteins inside the cell (Dodds, et al. 2004). The mature AVR proteins are highly polymorphic with at least one or more aa substitutions in the exposed surface of the proteins, suggesting functional interactions (Ellis, et al. 2007c, Wang, et al. 2007). Some of the isolates harbouring these AVR proteins became virulent on plants and overcame matching resistance genes, indicating that genes in the AvrL567 locus are under positive diversifying selection (Dodds, et al. 2006). The analysis of six flax rust isolates revealed twelve members of the AvrL567 gene family, including the three previously isolated AvrL567A-C genes and seven of these were avirulent while five were virulent and could no longer be recognized by L5, L6, or L7 (Dodds, et al. 2006). The *AvrM* gene family is a small family that consists of five avirulence paralogs, *AvrMA* to *AvrME*, and one virulent one, *avrM*, that is not recognized by any known flax R protein (Catanzariti, et al. 2006). These AVRM proteins do not have any known homologs and are highly variable both in sequence and size due to deletions or insertions of DNA, or to "pre-mature" termination of the protein because of the location of stop codons (Catanzariti, et al. 2006).

AVRP123 is a small cycteine-rich protein that contains the characteristic CX7CX6YX3CX2-3C signature of the kazal family of serine protease inhibitors, suggesting its role as an inhibitor of host proteases. This is similar to the function of AVR2 from *C. fulvum* that inhibits the RCR3 cysteine protease in the tomato apoplast (Catanzariti, et al. 2006). AVRP4 also encodes a small cyteine-rich protein of 67 aa after cleavage of a 28 aa signal peptide. The 28 aa C-terminal part of AVRP4 has six cysteine residues with the spacing consensus of a typical "cysteine knotted" peptide, similar to the *C. fulvum* AVR9 protein (van den Hooven, et al. 2001). *Avr*P4 is expressed only *in planta* while *AvrM* is expressed both *in planta* and *in vitro* (Stergiopoulos and de Wit 2009). Agroinfiltration of *AvrP4* and *AvrM* in flax plants with matching resistance genes results in a HR which indicates that these are functional in host cells and are, therefore, likely translocated into the host during infections; this is in agreement with the predicted cytoplasmic location of P and M resistance proteins in flax (Anderson, et al. 1997).

1.3.1e. *Magnaporthe oryzae*

M. oryzae (formerly known as *M. grisea*) is a filamentous ascomycete fungus that causes rice blast disease, destructive to rice production worldwide, but can also cause disease in many other members of gramineous plants (Couch and Kohn 2002, Kato, et al. 2000). More than forty resistance genes have been identified in rice against the blast fungus and several of them have been extensively used in resistant rice lines in the past few decades (Bryan, et al. 2000, Chen, et al. 2006). These resistant rice lines in the fields are overcome quickly by the emergence of new races of the pathogen through various mechanisms such as deletion of the *Avr* genes from the genome (Yoshida, et al. 2009) or changes in gene expression (Kang, et al. 2001, Fudal, et al. 2005) or point mutations in the *Avr* genes (Orbach, et al. 2000) resulting in escaping recognition by *R* genes; (Kolmer 1989, Leach, et al. 2001, McDonald and Linde 2002). Eight cultivar- and species-specific *Avr* genes have been cloned and characterized from *M. oryzae*: *Avr-Pita, Avr1-CO39, Ace1, Pwl1, Pwl2, AvrPiz-t, Avr-Pia, Avr-Pii* and *Avr-pik/km/kp* (Bohnert, et al. 2004, Collemare, et al. 2008, Farman and Leong 1998, Orbach, et al. 2000, Valent, et al. 1991, Li, et al. 2009, Miki, et al. 2009, Yoshida, et al. 2009). The AVR-PITA effector shows similarity to fungal metalloproteases of the deuterolysin family and is not required for full virulence on rice plants (Jia, et al. 2000, Orbach, et al. 2000). *Avr-Pita* encodes a 223 aa protein that is predicted to be secreted and processed into a 176 aa active form (AVR-PITA 176) that interacts with rice PITA resistance protein of the NBS-LRR class (Jia, et al. 2000). Only the processed form can trigger PITA-mediated defense responses by directly expressing it in rice cells (Jia, et al. 2000). Yeast two-hybrid assays and *in vitro* binding analyses showed a direct interaction between AVR-PITA 176 and the LRR of the PITA resistance gene protein (Jia, et al. 2000). In certain strains of *M. oryzae*, *Avr-Pita* undergoes spontaneous mutations in the laboratory and also under field conditions, such as deletion, point mutation, and the insertion of transposons, all resulting in overcoming *Pi-ta* resistance in rice cultivars (Kang, et al. 2001, Orbach, et al. 2000, Khang, et al. 2008, Zhou, et al. 2007). *Avr-Pita* is located close to the telomere of chromosome 3 in the genome of *M. oryzae* and this may be responsible for the genetic instability of this gene. *Avr-Pita* was renamed as *Avr-Pita1* after identification of *Avr-Pita2* and *Avr-Pita3* (Khang, et al. 2008). *Avr-Pita2* is recognized by the *Pita* gene from rice and elicits the defence response while *Avr-Pita3* is not recognized by PITA (Khang, et al. 2008).

Avr1-Co39 was isolated from *M. oryzae* isolate 4091-5-8 pathogenic on weeping lovegrass and specifies avirulence on rice cultivar CO39 that contains the resistant gene, *Pi-CO39*(t) in a gene for gene manner (Valent, et al. 1991, Chauhan, et al. 2002). The virulent isolates of *M. oryzae* on rice cultivar *CO-39* lack *Avr1-CO39* in most of the cases (Farman, et al. 2002). It has been shown that *M. oryza Avr1-CO39* is a species-specific rather than a cultivar-specific type of *Avr* gene (Zheng, et al. 2011). Another *Avr* gene, *Ace1*, encodes a 4035 aa polyketide synthase (PKS) fused to a nonribosomal peptide synthetase (NRPS); these are two different classes of enzymes that are probably involved in the production of a secondary metabolite that triggers Pi33-mediated resistance in rice cultivars (Bohnert, et al. 2004). *M. grisea* genome analysis revealed that *Ace1* is present in a cluster of 15 genes of which 14 encode enzymes such as a second PKS-NRPS (*Syn2*), two enoyl reductases (*Rap1* and *Rap2*) and a putative Zn(II)(2)Cys(6) transcription factor (BC2) which probably all play a role in secondary metabolism (Collemare, et al. 2008). *Ace1* and all other genes in the cluster are specifically expressed during penetration into the host plant, defining an infection-specific gene cluster, which suggests that *Ace1* might have a role in virulence; however, an *Ace1* disruption mutant did not show any reduction in virulence (Bohnert, et al. 2004, Fudal, et al. 2005). The *Pwl* genes isolated from a *M. oryzae* stop this pathogen from causing disease on weeping lovegrass and finger millet in a species-specific manner, but they still can infect rice (Kang, et al. 1995, Sweigard, et al. 1995). PWL effectors are small glycine-rich secreted proteins that are evolving fast and belong to a gene family designated as PWLI-PWL4. Virulent strains of *Pwl2* on weeping lovegrass appear due to spontaneous mutations, predominantly by genetic rearrangement and large deletions (Kang, et al. 1995, Sweigard, et al. 1995). In the three homologs of *Pwl2*, identified by homology searches, only *Pwl1* is the functional homolog while *Pwl3* and *Pwl4* are not functional; however, *Pwl4* is functional only when expressed under the control of the *Pwl1* or *Pwl2* promoter, while *Pwl3* is not functional in that case (Kang, et al. 1995). Recently, three new *Avr* genes, *Avr-Pia*, *Avr-Pii* and *Avr-Pik/Km/kp*, have been isolated from *M. oryzae* by association genetics (Yoshida, et al. 2009).

1.3.1f. *Leptosphaeria maculans*

L. maculans is an ascomycete fungus that causes blackleg (phoma stem canker) disease on oilseed rape (*Brassica napus)*. Genetic analysis of the interaction has revealed at least nine

avirulence genes designated as *AvrLm1-AvrLm9* that are recognized by corresponding resistance genes *Rlm1-Rlm9* of the host (Balesdent, et al. 2002, Fitt, et al. 2006, Rouxel and Balesdent 2005, Yu, et al. 2005). Seven of these nine genes are present in two unlinked clusters (*AvrLm1-2-6* and *AvrLm 3-4-7-9*) while the remaining two are individual genes (Balesdent, et al. 2002). *AvrLm1, AvrLm6* and *AvrLm4-7* have been cloned by a map-based strategy and all encode small putative secreted proteins and have no similarity to sequences in public databases (Fudal, et al. 2007, Gout, et al. 2006, Parlange, et al. 2009). *AvrLm1* has been cloned from this fungus and is clustered with two other *Avr* genes in the genome; *AvrLm6* has also been cloned from this cluster (Balesdent, et al. 2002, Gout, et al. 2006). Both *AvrLm1* and *AvrLm6* are located in a gene-poor, AT-rich, non-coding heterochromatin-like region as solo genes in stretches of 269 and 131 kb, respectively, which contain a number of degenerated nested copies of long terminal repeat (LTR) retrotransposon (Fudal, et al. 2007, Gout, et al. 2006). Also, both *AvrLm1* and *AvrLm6* are single copy genes that encode small proteins of 205 and 144 aa, respectively, with an N-terminal secretion signal but no other conserved motif or any similarity to each other (Fudal, et al. 2007, Gout, et al. 2006). The expression of both genes is strongly induced during early leaf infection but also expressed *in* the media at relatively low levels (Fudal, et al. 2007, Gout, et al. 2006). AVRLM1 has only one cysteine residues and is likely taken up by the host cell (Gout, et al. 2006), while AVRLM6 contains six cysteine residues that make disulphide bridges that could provide stability in the apoplastic environment (Fudal, et al. 2007). The *L. maculans* virulent strains on *Rlm1* oilseed rape cultivars lack the *AvrLm1* gene, like in other fungi. It has been shown that gain of virulence on *Rlm1* cultivars has been attained in 98% of the field isolates in France by deletion of an entire 260 kb locus (Gout, et al. 2006). Repeat-induced point (RIP) mutation and deletion were also responsible for gain of virulence on *Rlm1* cultivars (Fudal, et al. 2009). The *AvrLm4-7* gene was cloned via a map-based approach from a 238 kb genetic locus and is similar to the *AvrLm1* and *AvrLm6* loci by the presence of multiple LTR retrotransposon and a single gene in a gene-poor, AT-rich, 60 kb isochor (Parlange, et al. 2009). In this 238 kb region, a total of 40 predicted ORFs were identified of which 35 were in a "GC-equilibrated" region while only 5 were located in the AT-rich region (Parlange, et al. 2009). *AvrLm4-7* was the only gene in the 60-kb AT-rich region that was recognized by two resistance genes, *Rlm4* and *Rlm7* (Parlange, et al. 2009). *AvrLm4-7* encodes a cysteine-rich small secreted protein of 143 aa with a predicted N-terminal secretion signal of 21 aa (Parlange, et al. 2009). Like other effectors

from this fungus, the expression of *AvrLm4-7* is induced in planta but is also expressed at low levels in media (Parlange, et al. 2009). The partial or complete loss of *AvrLm4-7* in field isolates is responsible for gain of virulence on both *Rlm4* and *Rlm7* cultivars, while a point mutation that changes a glycine to an arginine residue, can overcome recognition by *Rlm4* (Parlange, et al. 2009).

1.3.2. Oomycete *Avr* genes

Oomycetes are filamentous fungus-like organisms that belong to the kingdom Stramenopila and are evolutionary related to algae. They are eukaryotic microorganisms and include some well-known pathogens of both cultivated and forest plants such as *P. infestans, H. arabidopsidis, P. sojae* and *P. ramorum*. Several effector proteins have been isolated and characterized from oomycetes (Table 1.1).

1.3.2a. *Hyaloperonospora arabidopsidis*

H. arabidopsidis, formerly known as *H. parasitica*, is an oomycete pathogen that causes downy mildew disease on the model plant *A. thaliana*. From this pathogen, two avirulence genes have been cloned and characterized, *Atr1*^{NdWsB} and *Atr13*Nd; the products are recognized by RPP1^{NdWsB} and RPP13Nd from *Arabidopsis* (Allen, et al. 2004, Rehmany, et al. 2005). ATR1, recognized by the products of two *Rpp1* genes from two *Arabidopsis* ecotypes, Niederzenze (Rpp1Nd) and Wassilewskkija (Rpp1^{WsB}), was identified by map-based cloning. RPP1Nd recognizes the product of a single allele of *Atr1*, while RPPI^{WsB} recognizes the products of four different alleles and provides resistance to a wide range of isolates (Rehmany, et al. 2005). *Atr1*^{NdWsB} encodes a 311 aa protein with a predicted secretion signal and a conserved RXLR motif that is present in most oomycete effectors (Rehmany, et al. 2005). Transient expression of *Atr1*^{NdWsB} in *Arabidopsis* leaves by particle bombardment triggered cell death (Kamoun 2006). ATR1^{NdWsB} is a highly polymorphic protein with six different alleles in eight isolates that differ in about one third of all residues (Kamoun 2006). Intense diversifying selection and recombination played an important role in the evolution of this locus (Kamoun 2006). *Atr13* encodes a 187 aa protein with no similar sequences found in public databases. It triggers a HR when transiently expressed by particle bombardment in *Arabidopsis* plants (Allen, et al. 2004). In addition to the N-terminal signal peptide and an RXLR motif, it has a heptad leucine/isolecine

repeat motif that is required for RPP13 recognition, followed by an imperfect direct repeat of 4 x 11 aa which lies between aa residues 93 and 136. ATR13 was apparently under intense diversifying selection and shows a high level of aa polymorphism, similar to it corresponding resistance protein, RPP13, suggesting a co-evolutionary arms race at these loci (Allen, et al. 2004). Both ATR1 and ATR13 suppress basal defense responses of host plants when delivered by the *P. syringae* DC3000 Type III secretion system, revealing their effector virulence function (Sohn, et al. 2007).

1.3.2b. *Phytophthora sojae*

P. sojae causes root and stem rot of soybean, resulting in huge damage to soybean production in North America (Shan, et al. 2004). Four avirulence genes designated as *Avr1b-1*, *Avr1a*, *Avr3a* and *Avr3c* have been cloned from this pathogen; they are recognized by soybean resistance genes *Rps1b, Rps1a, Rps3a* and *Rps3c* (Dong, et al. 2009, Qutob, et al. 2009, Shan, et al. 2004). *Avr1b-1* was cloned by map-based cloning and is predicted to encode a secreted protein of 138 aa with a RXLR motif and required another gene, *Avr1-b2*, at the locus for accumulation of *Avr1b-1* mRNA (Shan, et al. 2004). Virulent isolates of *P. sojae*, such as P6497 and P9073, harbour a complete *Avr1b-1* gene but cannot accumulate *Avr1b-1* mRNA like avirulent strains (Shan, et al. 2004). In addition to recognition by RPS1B, AVR1B-1 is also recognized by *Rpsk1* plants which can trigger limited cell death (Kamoun 2006). As effectors from the *Rpsk1* gene clusters are cytoplasmic, it is assumed that AVR1-B is recognized inside the host cytoplasm (Bhattacharyya, et al. 1997, Shan, et al. 2004).

P. sojae Avr1a, *Avr3a* and *Avr3c* were cloned by a combinatorial approach of genetic mapping, transcript profiling, and functional analysis (Qutob, et al. 2009). All *P. sojae* strains virulent and avirulent on *Rps1* plants contain the *Avr1a* gene that is present in four nearly identical copies of 5.2 kb but in some virulent strains, *Avr1a* is transcriptionally silenced (Qutob, et al. 2009). In other virulent strains, two fragments containing *Avr1a* are deleted (Qutob, et al. 2009). *Avr3a* also occurs in four duplicated copies of about 10.8 kb in addition to four other predicted ORFs located on the DNA fragment (Qutob, et al. 2009). Transcriptional silencing of these four copies of *Avr3a* is responsible for avoiding recognition by *Rps3* plants in some *P. sojae* virulent strains, while in other virulent strains three of the segments are deleted and the fourth one is transcriptionslly silenced (Qutob, et al. 2009). The *Avr3c* gene is present on a 33.7

kb fragment in addition to eight other predicted ORFs and three identical copies of this fragment are present in the *P. sojae* genome (Dong, et al. 2009). *Avr3c* virulent strains avoid recognition by RPS3A through specific mutations in the effector domain and subsequent sequence exchange between two copies of AVR3C (Dong, et al. 2009).

1.3.2c. *Phytophthora infestans*

P. infestans causes late blight disease in potato and tomato and was responsible for the 'Irish Famine' in 1840. Association genetics was used for the cloning of *Avr3a*, which encodes a cytoplasmic RXLR effector (Armstrong, et al. 2005). It is a secreted protein of 147 aa with two polymorphic residues in the mature protein (Armstrong, et al. 2005). $AVR3A^{K80/I103}$ ($AVR3A^{KI}$) is avirulent on potato *R3a*-expressing plants while the virulent allele has two aa substitutions $AVR3A^{E80/M103}$ ($AVR3A^{EM}$) (Armstrong, et al. 2005). $AVR3A^{KI}$ functions as an elicitor of defense in *R3a* plants, while in the absence of *R3a* it strongly suppresses *P. infestans* INF1 elicitin induced cell death in plants, thereby illustrating a major virulence function (Armstrong, et al. 2005, Bos, et al. 2009). The virulent allele, $Avr3a^{EM}$, cannot induce an *R3a*-mediated HR, but can weakly suppress INF1-induced cell death (Bos, et al. 2009, Bos, et al. 2006). These two different activities have been separated by making a series of mutants in important aa residues (Bos, et al. 2009). Deletions or mutations in the C-terminal residue, tyrosine 147, can maintain the R3A-mediated HR activity while it causes the loss of the ability to suppress INF1-induced cell death (Bos, et al. 2009, Bos, et al. 2006)). Also, $AVR3A^{KI}$ interacts and stabilizes the host ubiquitin E3-ligase CMPG in host plants which is required for INF1-induced cell death (Bos, et al. 2010). Silencing of *Avr3a* reduces pathogenicity which means it is required for full virulence. Transient expression of *Avr3a* in plants without a signal peptide induces *R3a*-mediated HR and, therefore, this effector is considered to be recognized in the plant cytoplasm (Armstrong, et al. 2005). Avr3a of *P. infestans* is located in a syntenic region to *H. arabidopsidis* $ATR1^{NdWsB}$, suggesting that this locus is ancient in these oomycetes (Armstrong, et al. 2005).

1.4. Resistance proteins (R) in plants

AVR proteins are delivered either to the apoplast, the cytoplasm, or to the nucleus of plant cells depending on their target. This is consistent with the location of matching R proteins. For

example, most of the bacterial effector proteins are injected directly into the cytoplasm by the Type III secretion system and the corresponding R proteins to most of these effector proteins are also intracellular (Staskawicz, et al. 2001). Some nematodes, oomycetes, and fungi also translocate their AVR proteins to the cytoplasm (Bryan, et al. 2000, Dodds, et al. 2004, Dodds, et al. 2006, Jia, et al. 2000, Orbach, et al. 2000). On the other hand, the AVR2, AVR4, AVR4E and AVR9 proteins of *C. fulvum*, are secreted into the apoplast (Joosten and de Wit 1999, Lauge, et al. 1998) which is consistent with the membrane localization of their matching CF proteins (Piedras, et al. 2000). The AVRBS3-like protein has a nuclear localization signal (NLS), which would suggest that its corresponding R protein is located in the nucleus (Lahaye and Bonas 2001).

Several functional *R* genes encoding resistance against bacterial, fungal, viral, oomycete, nematode, and insect pathogens have been isolated from various model and crop plants. This wide range of R proteins can be divided into five or six classes on the basis of domain structure (Dangl and Jones 2001, Martin, et al. 2003, Nimchuk, et al. 2003). Class I has only one member, PTO from tomato, which has a myristylation site at the N-terminal end and a serine/threonine kinase catalytic activity domain. The majority of the R proteins are Nucleotide Binding Site-Leucine Rich Repeat (NBS-LRR) proteins, which make up classes II and III of R proteins. The only difference between these two classes is located in the N-terminal region of their members. Class II members have a coiled-coil domain and class III members have a Toll or Interleukin 1 Receptor (TIR) domain at the N-terminal end. Proteins in all these three classes are intracellular, as they do not have any predicted transmembrane domains. R proteins in classes IV and V are similar to each other in the sense that both have extracellular LRRs and a transmembrane domain, but class IV members have a short cytoplasmic tail with unknown function. Class V members have a cytoplamic serine/threonine kinase domain like PTO. Resistance proteins such as HM1, RPW8, MLO, and several others do not easily fit into any of the above mentioned classes and are put into class VI.

1.5. Recognition of Avr proteins by R proteins

The recognition of invading pathogens is a critical step in the activation of the host defense response by R proteins. Plant resistance proteins use either a direct or an indirect mode of

recognition of specific elicitors from pathogens. In the direct recognition systems, the AVR protein acts as a ligand and the R protein as a receptor. For example, based on yeast two-hybrid screens, the rice *Pita R* gene product binds the cognate AVR-PITA from *M. grisea* (Jia, et al. 2000). Similarly, RRS1-R from *Arabidopsis* binds POPP2 from *R. solanacearum* (Deslandes, et al. 2003). Direct recognition has been shown to operate in recognition of AVRL567 from *M. lini* by flax L5, L6 and L7 (Dodds, et al. 2006) Dodds PN 2006). In indirect recognition systems, the R protein recognizes the pathogen effectors through detection of changes in their host protein target (Van der Biezen and Jones 1998). This suggested model is called the "guard model" in which the R protein guards the effector target, also called guardee, and can then detect changes in the guardee. This seems to be a more common recognition system than the receptor-ligand system (Jones and Dangl 2006). Several studies of bacterial Type III secretion system effector proteins and their corresponding R proteins support the guard model. The RPM1 protein in *Arabidopsis* recognizes effector proteins AVRRPM1 and AVRB from *P. syringae* indirectly by changes in the host protein RIN4 (Mackey, et al. 2002). Both of these Type III effectors induce phosphorylation of RIN4, which serves as a signal for activation of RPMI. Changes in RIN4 are also perceived by another protein, RPS2, when the pathogen delivers AVRRPT2, a cysteine protease, inside the cell which cleaves RIN4 (Day, et al. 2005, Mackey, et al. 2003). RIN4 interacts with another protein, NDR1, which is required for the activation of RPM1 and RPS2 (Day, et al. 2006). AVRRPT2 causes degradation of RIN4 at two different sites through its cysteine protease activity. The down-regulation of RIN4 activates RPS2 (Chisholm, et al. 2005, Kim, et al. 2005). RIN4 is not the only target of these effector proteins in the host, as AVRRPT2 can degrade several other *Arabidopsis* proteins that contain its corresponding cleavage site (Chisholm, et al. 2005). It means that the contribution of an effector to virulence might involve several host targets and the generation of several modified host molecules, but the perturbation of only one is sufficient for activation of the *R* gene (Belkhadir, et al. 2004, Day, et al. 2005). In *rpm1 rps2* double mutants, AVRRPT2 and AVRRPM1 manipulate RIN4 for suppressing PAMP-triggered immunity (Dodds, et al. 2006). RPS5 from *Arabidopsis* monitors changes in PBS1, which is targeted by AVRPPHB of *P. syringae* (Shao, et al. 2002). CF-2 in tomato monitors changes in RCR3 brought about by the activity of *C. fulvum* AVR2 (Chang, et al. 2000). Two effectors, EPIC1 and EPIC2B from *P. infestans*, bind to RCR3 that plays a role in defense against *P. infestans* (Dangl and Jones 2001). Similarly, tomato PRF monitors changes in

PTO caused by AVRPTO and AVRPTOB from *P. syringae* (Jones and Dangl 2006, Van der Biezen and Jones 1998). Recently, the decoy model has been proposed for the indirect recognition of AVR proteins by R proteins, based on a lack of evidence that guardees increase host susceptibility in the absence of their matching R proteins (van der Hoorn and Kamoun 2008). In the decoy model, the target of the effector is required for the function of the R protein but it does not have any direct function in host susceptibility or resistance (van der Hoorn and Kamoun 2008).

Whether R and AVR proteins interact directly or indirectly, the ultimate result is an incompatible interaction between plant and pathogen, usually associated with HR. The HR in plants directed against microbial pathogens is a type of rapid and localized programmed cell death (PCD) similar to that in mammalian tissues and differs from developmental PCD only in its consistent association with the induction of local and systemic defence responses. The number of dead cells varies from one to dozens and depends on both genotypes of pathogen and plants. This HR in plants is usually visible macroscopically, but in some incompatible plant-pathogen interactions, such as the *Ustilago hordei*-barley interaction, it can be seen only microscopically (Hu, et al. 2002); however, there are also a few other reported cases of gene-for-gene resistance that do not produce any visible HR, such as the *Arabidopsis dndI* (defense no death) mutant, which produces resistance in a gene-for-gene manner without any visible HR against avirulent bacteria (Yu, et al. 1998). Similarly, the *Rx* gene of potato recognizes the potato virus *X* gene in a gene-for-gene manner but does not produce any microscopic or macroscopic HR lesions on potato plants or transgenic tobacco plants (Kang, et al. 1995).

1.6. Marker-based approaches for cloning of *Avr* genes

Most known pathogen *Avr* genes are highly diverged in DNA sequence and many do not have annotated homologs in public databases, although similar sequences can often be found in the genomes of related species. Identifying functional *Avr* genes has been a challenge (Gan, et al. 2010b, Van't Slot and Knogge 2002). In the case of bacterial phytopathogens, several *Avr* genes have been isolated by classical genetic techniques commonly used for bacterial gene isolation, such as transformation of a genomic library from an avirulent strain into a virulent strain and subsequently testing for a HR response on host plants to select for *Avr*-containing clones

(Collmer 1998, Van den Ackerveken and Bonas 1997). This method is not easily applicable for isolating *Avr* genes from fungi and oomycetes due to large genome sizes and inefficient transformation methods in many cases (Lauge, et al. 1998). Reverse genetics and map-based cloning are the two main strategies that have been successfully used for isolating fungal and oomycetes *Avr* genes. A reverse genetics approach is based on the isolation and purification of proteins encoded by *Avr* genes that elicit the defense response in specific resistant cultivars or nonhost plants. Using this approach, several fungal *Avr* genes have been isolated, such as *Nip1* from the barley leaf scald pathogen, *R. secalis* (Rohe, et al. 1995), *Avr9* (van Kan, et al. 1991), *Avr4* (Joosten, et al. 1994), *Ecp1, Ecp2* (Van den Ackerveken, et al. 1993), *Ecp3* and *Ecp4* (Lauge, et al. 2000) from *C. fulvum*, *AvrM, AvrP4, AvrP123* and *AvrL567* from *M. lini* (Catanzariti, et al. 2006, Dodds, et al. 2006), and *Avr2* (Houterman, et al. 2007), *Avr3* (Rep, et al. 2004) and *Avr1* (Houterman, et al. 2008) from *F. oxysporum*. Similarly, a few oomycete *Avr* genes have been isolated by this approach such as *Inf1* from *P. infestans* (Kamoun, et al. 1998) and *Gip1* and *Gip2* from *P. sojae* (Rose, et al. 2002).

For intracellular pathogens such as *M. oryzae*, a reverse genetics approach was not very successful for isolating *Avr* genes. *M. oryzae Avr* genes such as *Pwl2* (Sweigard, et al. 1995), *AvrPita* (Farman and Leong 1998), *AvrCo39* (Miki, et al. 2009) and *Avr-Pia* (Miki, et al. 2009, Chen, et al. 2007) were isolated by positional cloning strategy. In addition, a few *Avr* genes, *AvrPi15* (Ma, et al. 2006) and *Pre1* (Miki, et al. 2009), have been mapped on a short genetic interval by linkage mapping in *M. oryzae*. Similarly, *AvrLm6* (Fudal, et al. 2007), *AvrLm4-7* (Parlange, et al. 2009) and *AvrLm1* (Gout, et al. 2006) from *L. maculans, Avra10* and *Avrk1* from *B. graminis* f. sp. *hordei* (Ridout, et al. 2006) were isolated by map-based cloning and *Avra22* and *Avra12* from *B. graminis* f. sp. *hordei* were mapped on short genetic intervals (Skamnioti, et al. 2008). Several oomycete *Avr* genes have been isolated by map-based cloning, such as *Avr1b-1* and *Avr1b-2* (Shan, et al. 2004), *Avr1a* (Qutob, et al. 2009) and *Avr3c* (Dong, et al. 2009) from *P. sojae,* and *Atr1* (Rehmany, et al. 2005) and *Atr13* (Allen, et al. 2004) from *H. arabidopsidis*. Relevant to this thesis, *UhAvr1* was mapped to an 85 kb genetic interval in the barley covered smut fungus, *U. hordei*, by a marker-based approach (Linning, et al. 2004). Several other techniques have been employed, including using bioinformatics and association genetics. *Avr-Pia, Avr-Pii* and *Avr-Pik/km/kp* were isolated from *M. oryzae* (Yoshida, et al. 2009) and *Avr3a* from *P. infestans* (Armstrong, et al. 2005). *Avr3a* was identified in *P. sojae* by correlating gene

transcript profiling data with phenotypically pooled progeny using microarrays (Qutob, et al. 2009). Stergiopoulos et al. (2010) isolated orthologs of *Avr4* and *Ecp2* from *M. fijiensis*, the causal agent of black sigatoka disease of banana, by means of a bioinformatic homology search approach.

1.7. Comparative genomics (secretomics)

The continuous arms race between pathogens and their host plants has intensively affected the co-evolution of pathogen and plant genomes. Whole genome sequencing and analyses show that *R* genes are the most polymorphic genes as compared to the rest of the genome (Clark, et al. 2007). Similarly, effector genes from pathogens are also evolving at a fast pace and in few cases, these effectors are present on unstable parts of the genome such as at telomeres, or reside on small, dispensible chromosomes (Gout, et al. 2006, Orbach, et al. 2000). Comparative genomics is a powerful tool and can be very useful for the identification of new virulence and avirulence effectors from phytopathogens by comparing the genome sequences of closely related pathogens. Several clusters of effectors have been identified by comparing the genomes of two related basidiomycete smut fungi, *U. maydis* and *Sporisorium reilianum*, both infecting corn (Schirawski et al. 2010). In *M. oryzae*, 316 new candidate effectors have been identified by genome comparison of isolate 70-15 and a field isolate, Ina 168 (Yoshida, et al. 2009), and three of them have proved to be *Avr* genes. The effectors from oomycetes pathogens have a host-targeting RXLR-dEER motif, in addition to a secretion signal at the N-terminal end (Tyler 2009). The genome sequences of *P. sojae, P. ramorum, P. infestans*, and *H. arabidosidis* have a large number of RXLR-dEER effectors with 40-50 % identity across species (Jiang, et al. 2008). The majority of effectors from different cellular pathogens are predicted to be secreted or have been proven to be secreted by different mechanisms (Chisholm, et al. 2006, Huang, et al. 2003, Kamoun 2007). The effector proteins of certain oomycetes and fungi can be largely predicted computationally (Ellis, et al. 2007b, Kamoun 2007). Because many fungal and oomycete genomes have been sequenced or are close to completion, an opportunity exists to predict a complete suite of secreted proteins. The processing of these sequences by tools of comparative genomics (Tyler, et al. 2006) and *in silico* prediction of secreted proteins (Torto, et al. 2003) has

identified a substantial number of candidate effector genes that could be involved in pathogenesis.

1.8. Smut fungi

The work presented in this thesis involves the experimental organism, the barley covered smut fungus, *U. hordei*. Smut fungi belong to Order Ustilaginales of the basidiomycetes and occur throughout the world. There are approximately 1400 species of smut fungi in over 70 genera (Agrios 2005, Fisher and Holton 1957). These are facultative obligate biotrophs that cause diseases in 4,000 species of angiosperms belonging to approximately 75 different families. The genus, *Ustilago*, mainly infects cereal crops and grasses belonging to the Gramineae family, which are used as food and feed. Most smut fungi infect the ovaries of grains and grasses and destroy the fruits (kernels) of grains, completely; however, some smuts can infect and produce spores on vegetative and floral parts, such as the corn smut, *U. maydis* (Alexopoulos, et al. 1996, Fisher and Holton 1957). Usually, smut fungi do not kill their hosts but in some cases, the diseased plant may be severely stunted. Most of the smut diseases can be controlled by treatment of seeds with fungicides or the use of resistant varieties.

The fungal pathogen, *U. hordei*, causes covered smut on barley and oats and not only decreases the yield of the crops, but also greatly reduces the quality of the remaining yield due to the presence of black smut spores on the surface of healthy kernels. *U. hordei* is a representative of smut fungi that infect small grains. This fungus infects the seedling and grows as dikaryotic hyphae within the developing plant without any visible symptoms until flowering. During flowering, the fungal cells proliferate, making thick-walled teliospores during which karyogamy takes place (Hu, et al. 2002). Teliospores disseminate from the diseased head and contaminate healthy barley seeds of nearby plants and usually overwinter under the seed hull. When the conditions are favorable for germination, both seed and teliospores germinate. Upon germination, teliospores undergo a process of meiosis giving rise to haploid basidiospores that segregate 1:1 for mating types *MAT-1* and *MAT-2* (Bakkeren and Kronstad 1994). The basidiospores multiply by budding and are amenable to different types of molecular genetic techniques. The opposite mating types, *MAT-1* and *MAT-2*, can recognize each other by a pheromone/receptor system (Bakkeren and Kronstad 1996), forming dikaryotic hyphae by

fusion. These resulting dikaryotic hyphae can penetrate emerging seedlings by direct penetration (Hu, et al. 2002), thereby completing the life cycle (Figure 1).

The smuts are important pathogens that cause disease world-wide, resulting in crop losses in many countries, including Canada (Menzies, et al. 1996, Thomas 1989). The barley/*U. hordei* pathosystem is an excellent model system for small grain-infecting basidiomycetes due to the presence of race cultivar-, 'gene-for-gene'-based resistance and the availability of resources such as a genetic transformation system, gene deletion techniques, genetic crosses, many field isolates and races, and several differential barley cultivars. Also, the complete genome sequence of *U. hordei* became available during the course of my thesis work (collaborative effort with a group from the Max Planck Institute for Terrestrial Microbiology in Marburg, Germany: Drs. J. Schirawski and R. Kahmann), and complete genome sequence data and many ESTs are available from the closely related fungi, *U. maydis* and *S. reilianum* (Kamper, et al. 2006, Schirawski, et al. 2010). Six *Avr* genes have been described in *U. hordei* which in different combinations constitute fourteen different reported races (Tapke 1945). Six corresponding resistance genes have been identified in barley (Thomas 1976). At the onset of my thesis work, a population of 54 progeny, resulting from a cross between two parents possessing dominant and recessive avirulence genes and segregating genetically for three *Avr* genes, *UhAvr1, UhAvr2* and *UhAvr6*, were available in our laboratory (Linning, et al. 2004). Two dominant genes, *UhAvr1* and *UhAvr6,* act consistently in a stable genetic manner while *UhAvr2* expression is influenced by environmental conditions (Linning, et al. 2004). The *UhAvr1* gene has been identified on a genomic region of 85 kb (Laurie and Bakkeren, unpublished). In addition, BAC libraries from both the virulent and avirulent parents of the mapping population and a physical BAC map for this pathogen were available in our laboratory (Bakkeren, et al. 2006).

1.9. Proposed research project

The isolation and characterization of *Avr* genes from pathogens and *R* genes from their hosts is an important focus of research in molecular plant pathology to understand the biochemical and molecular basis of effector-triggered immunity in plants (Hammond-Kosack and Parker 2003). This will help us to understand the compatibility and disease potential, suppression of host defenses, functions of effectors as elicitors, the host targets of these effectors, virulence of the

pathogen, and defense initiation. The cloning and characterization of *Avr* genes is made feasible because they are single genes and are usually dominant, thus making them easy to follow genetically. Currently, our molecular genetic knowledge of fungal *Avr* genes is based on only seven of the above-mentioned fungal species. Most of these *Avr* gene sequences are much diverged from one another and also do not have many similar sequences in public databases (Gout, et al. 2006). *M. oryzae*, an ascomycete, is the only fungal pathogen infecting cereal crops from which *Avr* genes have been characterized (Chen, et al. 2007, Farman and Leong 1998, Miki, et al. 2009, Sweigard, et al. 1995). *M. lini*, the flax rust fungus, is the only basidiomycete from which *Avr* genes have been characterized (Catanzariti, et al. 2006, Dodds, et al. 2004, Dodds, et al. 2006). No *Avr* genes have been isolated from basidiomycetes infecting monocots thus far. To fill this gap, we need to build a model system to study the molecular interaction of *R* and *Avr* genes in basidiomycetes and monocots to which all cereal crops belong. *U. maydis*, the corn smut, is a widely-studied pathogen with a sequenced genome but no *Avr* genes have been described (Kamper, et al. 2006). *U. hordei* and barley offer an excellent model system in this regard to understand the *Avr-R* gene interaction between basidiomycetes and monocots. *UhAvr1* has been isolated on a 85-kb region by a marker based approach (Linning, et al. 2004). The corresponding resistance gene *UhR1* has been mapped to the short arm of chromosome 1 in barley by our collaborators at the University of Saskatchewan (Grewal, et al. 2008). Barley cultivar Morex that contains the *Ruh1* gene, is available on a BAC library (Dr. A. Kleinhofs, Washington State University; (Brueggeman, et al. 2002) and a barley cDNA library in a yeast two-hybrid specific vector is available (Dr. R. Hueckelhoven, University of Giessen, Germany), allowing for follow-up research involving *R* gene and effector target studies. Studying the interaction between *UhAvr1* and *Ruh1* will help to understand the mechanism of pathogenesis and elicitation of the defense response. Identifying *UhAvr1*, the analysis of the effector locus might shed some light on mechanisms involved in its evolution since, as I will show, these *Ustilago* effector loci are somewhat conserved between *U. hordei* and *U. maydis*. My research project was mainly focused on identifying and characterizing the *UhAvr1* gene on the 85-kb region of *U. hordei* genomic DNA. I also used comparative genomics to understand the evolution of this locus between two related smut fungi. Pathogen effectors are rapidly evolving and in many cases, pathogen avirulence genes are present in regions displaying high genome flexibility, such as telomeric (Orbach, et al. 2000), or heterochromatic locations (Fudal, et al. 2007, Parlange, et al. 2009), or

they are surrounded by transposable elements (Fudal, et al. 2007, Gout, et al. 2006, Khang, et al. 2008, Zhou, et al. 2007). I was therefore interested in finding out in what sort of molecular environment the *UhAvr1* gene is located and what the molecular basis was of the change from avirulent *U. hordei* strains to overcome *UhR1* resistance in the field.

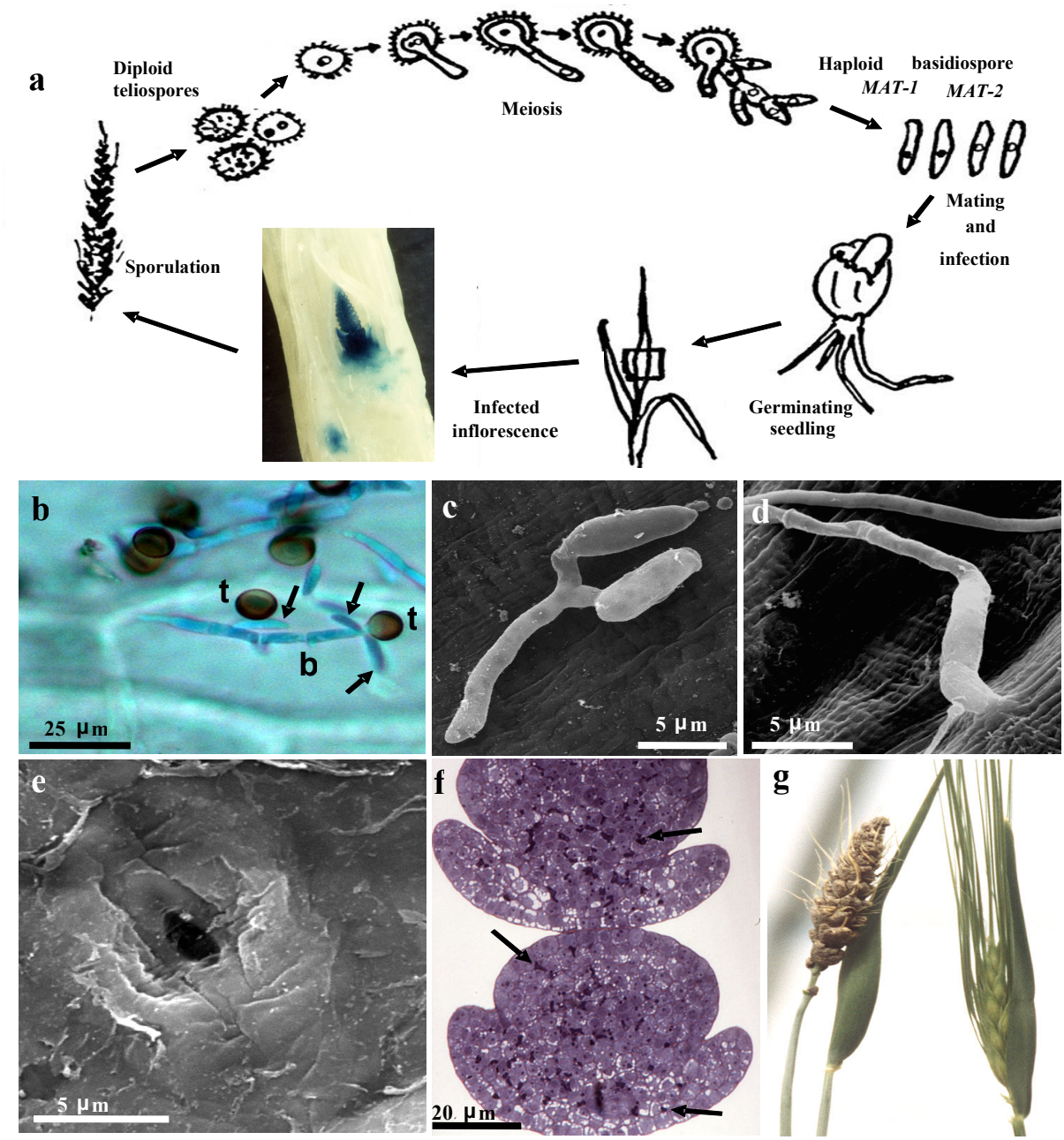
I was also interested in other small secreted proteins (SSPs) of *U. hordei* that may have virulence or avirulence functions. For this purpose, I searched for *U. hordei*-specific SPs by comparing all predicted SPs from the *U. hordei* genome to the predicted suites of SPs from *U. maydis* (Kamper, et al. 2006, Schirawski, et al. 2010) using bioinformatic approaches. In the population of *U. hordei*, an additional *Avr* gene, *UhAvr6*, was segregating; this gave me the opportunity to work towards the cloning of this gene as well. For the cloning of *UhAvr6*, I used SSR, RAPD and AFLP techniques to find molecular markers linked to this gene. A collaborator tested *U. hordei* strains on new barley lines and found potential new *Avr* genes and corresponding *R* genes. In order to initiate cloning of this gene(s) as well, I developed a *U. hordei* population segregating for these new *Avr* genes.

The genetic transformation system for *U. hordei* is not very efficient and does not easily allow for the transfer of large genomic fragments. The current method of protoplast transformation is dependent on the use of lytic enzymes which need to be optimized for each batch of enzyme. This makes this method inconsistent. Since many effectors reside in paralogous clusters on large genomic fragments, the transfer of such regions into recipient *U. hordei* strains would possibly allow for functional complementation analyses. *Agrobacterium* mediated transformation (AMT) has been used efficiently for several filamentous fungi and worked better for several fungi that were difficult to transform by traditional transformation methods (Chen, et al. 2000, Degefu and Hanif 2003, Meyer, et al. 2003, Mikosch, et al. 2001, Michielse, et al. 2005b). In addition to its efficiency, AMT results in transformation of single copy integration of the T-DNA at random sites in the genome making it suitable for insertion mutagenesis (Combier, et al. 2003, Mullins, et al. 2001, Takahara, et al. 2004). I therefore developed an AMT-based transformation protocol for *U. hordei*. To subsequently be able to transfer to *U. hordei* large genomic fragments without the need for cumbersome cloning with restriction and ligation enzymes, I adapted a technique to engineer *Agrobacterium* binary vectors by *in vitro* recombineering so these vectors can contain BAC clones (BIBACs).

The knowledge obtained from my dissertation research will not only be helpful in understanding molecular interactions between plant and pathogen but will also be helpful in designing novel ways to increase crop resistance.

1.10. Research objectives

1. Identification and characterization of the *Ustilago hordei* avirulence gene 1 within the *UhAvr1* locus, and possibly *UhAvr6*, to extend our knowledge of effector-triggered immunity in plants to now include an interaction between a basidiomycete pathogen and a monocot host, and to further our knowledge of fungal effectors in disease establishment and defense induction
2. To study how *U. hordei* overcomes *Ruh1*-triggered resistance in barley
3. To understand evolutionary pressures acting on the *UhAvr1* locus in light of its similarity to a locus in the closely-related corn smut fungus, *U. maydis*
4. To gain insight into the potential repertoire of small secreted proteins (effectors) of *U. hordei* likely involved in virulence and avirulence towards the host plant
5. To study the function of clusters of predicted secreted proteins by developing a reliable technique for introducing large genomic DNA fragments into *U. hordei*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Diploid
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Haploid basidiospore
MAT-1 MAT-2
Mating
and
infection
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20 µm
g

Figure 1.1 Infection process of *U. hordei* on barley. a. schematic representation of the *U. hordei* life cycle. Dispersed teliospores become lodged under seed hulls and germinate together with the seed; mating needs to precede infection which can only occur on the young coleoptile. Photographic insert depicts a light microscopic picture of an immature inflorescence at 5 weeks after infection showing blue-colored fungi expressing the β-glucuronidase gene after treatment with glucuronide. b. light microscopic picture of germinated teliospores (t) on the surface of a barley coleoptile 17 hrs after inoculation having produced a basidium (b) from which haploid basidiospores (arrows) are emerging (cotton blue-staining). c. scanning electron micrograph of two mated cells of opposite mating type fused through conjugation hyphae to produce the dikaryotic infection filament on a barley coleoptile. d. SEM of a dikaryotic infection hypha entering the barley coleoptile wall by direct penetration. e. SEM showing a penetration site from which the hypha has been removed. f. light microscopic picture of an immature inflorescence showing extensive, early teliospores formation (arrows). g. emerged head where all kernels have been replaced by black teliospores, next to a healthy head (see Hu et al. 2002, for details).ced by black teliospores, next to a healthy head (see Hu et al. 2002, for details).

Table 1.1 Effector proteins of filamentous plant pathogens

Protein	Organism	Length aa residues (mature)	No: of Cysteines	Signal Peptide in aa	Biological activity/homology	Protein localization	Role in virulence/pathogenicity	Corresponding R-gene	References
Avr2	*C. fulvum*	78 (58)	8	20	induces HR in the presence of Tomato Rcr3, Protease inhibitor,	Apoplast	Inhibits Rcr3 and other proteases	*Cf-2*	(Stergiopoulos, et al. 2010)
Avr4	*C. fulvum*	135 (86)	8	18	induces HR, Chitin-binding, orthologs in some other fungi	Apoplast; Fungal cell wall chitin	Protects against chitinases	*Cf-4*	(Joosten, et al. 1994, Stergiopoulos, et al. 2010) (van den Burg, et al. 2006)
Avr4E	*C. fulvum*	121 (101)	6	10	induces HR,	Apoplast	Unknown	*Hcr9-4E*	(Westerink, et al. 2004)
Avr9	*C. fulvum*	63 (28)	6	23	induces HR, Carboxypeptidase inhibitor	Apoplast	Unknown	*Cf-9*	(Van den Ackerveken, et al. 1993)
Ecp1	*C. fulvum*	96 (65)	8	23	induces HR, Tumor-necrosis factor receptor	Apoplast	Disruption leads to reduced virulence	*Cf-Ecp1*	(Van den Ackerveken, et al. 1993)
Ecp2	*C. fulvum*	165 (101)	4	22	induces HR,	Apoplast	Disruption leads to reduced virulence	*Cf-Ecp2*	(Van den Ackerveken, et al. 1993)
Ecp4	*C. fulvum*	119 (101)	6	18	induces HR,	Apoplast	Unknown	*Cf-Ecp4*	(Bolton, et al. 2008)
Ecp5	*C. fulvum*	115 (98)	6	17	Induce necrosis,	Apoplast	Unknown	*Cf-Ecp5*	(Bolton, et al. 2008)
Ecp6	*C. fulvum*	222 (199)	8	23	LysM-domains; chitin-binding, ortholog found in different pathogen and non-pathogenic sp.	Apoplast	Knock-down leads to reduced virulence	Unknown	(de Jonge and Thomma 2009)

Protein	Organism	Length aa residues (mature)	No: of Cysteines	Signal Peptide in aa	Biological activity/homology	Protein localization	Role in virulence/pathogenicity	Corresponding R-gene	References
Ecp7	*C. fulvum*	- (100)	6	-	Unknown	Apoplast	Unknown	Unknown	(Bolton, et al. 2008)
Nip1	*R. secalis*	82 (60)	10	22	Non-specific toxin/induces necrosis and plasma-membrane H+ ATPase	Probably in apoplast	Not required for virulence	*Rrs-1*	(Rohe, et al. 1995)
Nip2	*R. secalis*	109 (?)	7 (6)	16	Non-specific toxin/induces necrosis in several plants species	Probably in apoplast	Not required for full virulence	Unknown	(Rohe, et al. 1995) (Stergiopoulos and de Wit 2009)
Nip3	*R. secalis*	115 (?)	9 (8)	17	Non-specific toxin/induces necrosis in several plants species,	Probably in apoplast	Not required for full virulence	Unknown	(Rohe, et al. 1995) (Stergiopoulos and de Wit 2009)
Avra10	*B. graminis*	286	4	-	More than 30 paralogues in *Bgh* and other f. sp. No N-terminal SP, induces HR,	Probably in cytoplasm	Unknown	*Mla10*)	(Ridout, et al. 2006)
Avrk1	*B. graminis*	177	3	-	More than 30 paralogues in *Bgh* and other f. sp. No N-terminal SP, induces HR,	Probably in cytoplasm	Unknown	*Mlk1*)	(Ridout, et al. 2006)
AvrL567 (A, B and C)	*M. lini*	150 (127)	1	23	Unknown induces HR, Functional RXLR like motif	Cytoplasm	Unknown	*L5*, *L6* and *L7*	(Dodds, et al. 2004) (Kale, et al. 2010) (Rafiqi, et al. 2010)

Protein	Organism	Length aa residues (mature)	No: of Cysteines	Signal Peptide in aa	Biological activity/homology	Protein localization	Role in virulence/pathogenicity	Corresponding R-gene	References
AvrM	*M. lini*	314	1	28	Unknown, induces HR, RXLR like motif	Cytoplasm	Unknown	*M*	(Catanzariti, et al. 2006) (Kale, et al. 2010) (Rafiqi, et al. 2010)
AvrP123	*M. lini*	117 (94)	11	23	induces HR,, Kazal Ser protease inhibitor	Probably in cytoplasm	Unknown	*P*, *P1*, *P2* and/or *P3*)	(Catanzariti, et al. 2006))
AvrP4	*M. lini*	95 (67)	7	28	induces HR, Cystine knotted peptide	Probably in cytoplasm	Unknown	*P4*	(Catanzariti, et al. 2006)
Avr-Pita	*M. oryzae*	224 (176)	8	16	Homology to Metalloproteases, RXLR like motif	Cytoplasm	Not required for virulence on rice	*Pi-ta*	Orbach, MJ 2000, (Kale, et al. 2010), (Khang, et al. 2010)
Avr-Pita2	*M. oryzae*	224 (?)	8	16	Homology to Metalloproteases	Probably in apoplast	Probably not required for virulence on rice	*Pi-ta*	(Khang, et al. 2008)
Avr-Pita3	*M. oryzae*	226 (?)	8	16	Homology to Metalloproteases	Probably in cytoplasm	Probably not required for virulence on rice	Unknown	(Khang, et al. 2008)
Pwl1	*M. oryzae*	147 (124)	2	23	Glycine-rich hydrophilic protein	Biotrophic interfacial complex	Unknown	Unknown	(Kang, et al. 1995) (Khang, et al. 2010)
Pwl2	*M. oryzae*	145 (126)	2	21	Glycine-rich hydrophilic protein	cytoplasm	Unknown	Unknown	(Sweigard, et al. 1995) (Khang, et al. 2010)
Pwl3	*M. oryzae*	137 (116)	0	21	Glycine-rich hydrophilic protein	Probably in apoplast	Non-functional	Unknown	(Kang, et al. 1995)

Protein	Organism	Length aa residues (mature)	No: of Cysteines	Signal Peptide in aa	Biological activity/homology	Protein localization	Role in virulence/pathogenicity	Corresponding R-gene	References
Pwl4	*M. oryzae*	138 (117)	0	21	Glycine-rich hydrophilic protein	Probably in apoplast	Non-functional	Unknown	(Kang, et al. 1995)
Ace1	*M. oryzae*	4035	43	-	Hybrid polyketide synthase/nonribosomal peptide synthetase	Not secreted	Unknown	*Pi33*	(Bohnert, et al. 2004)
Avr1-CO39	*M. oryzae*	Not cloned yet	-	-	Unknown	Unknown	Unknown	*Pi-CO39(t)*	(Farman, et al. 2002)
Avr-Pia			2		induces HR	Probably in cytoplasm	Unknown		(Yoshida, et al. 2009)
Avr-Pii			3		induces HR	Probably in cytoplasm	Unknown		(Yoshida, et al. 2009)
Avr-Pik/km/kp			3		induces HR	Probably in cytoplasm	Unknown		(Yoshida, et al. 2009)
AvrLm1	*L. maculans*	205 (183)	1	22	induces HR,	Probably in cytoplasm	Required for full virulence	*Rlm1*	(Gout, et al. 2006)
AvrLm6	*L. maculans*	144 (124)	6	20	Unknown, Functional RXLR like motif	Probably in apoplast	Unknown	*Rlm6*	(Fudal, et al. 2007) (Kale, et al. 2010)
AvrLm4-7	*L. maculans*	143 (122)	8	21	Unknown	Probably in apoplast	Required for full virulence	*Rlm4* and/or *Rlm7*	(Parlange, et al. 2009)
Avr3 (Six1)	*F. oxysporum* f. sp. *hordei*	284 (189)	6 or 8	21	Unknown	Xylem	Required for full virulence	*I-3*	(Rep, et al. 2004) (Stergiopoulos and de Wit 2009)

Protein	Organism	Length aa residues (mature)	No: of Cysteines	Signal Peptide in aa	Biological activity/homology	Protein localization	Role in virulence/pathogenicity	Corresponding R-gene	References
Avr4 (Six2)	*F. oxysporum* f. sp. *hordei*	232 (172)	8	20	Unknown	Xylem	Probably not required for virulence	Unknown	(Houterman, et al. 2007) (Stergiopoulos and de Wit 2009)
Avr2 (Six3)	*F. oxysporum* f. sp. *hordei*	163 (144)	3 (2)	19	induces HR, Unknown, Functional RXLR like motif	Xylem	Required for full virulence	*I-2*	(Houterman, et al. 2007) (Kale, et al. 2010, Stergiopoulos and de Wit 2009)
Avr1 (Six4)	*F. oxysporum* f. sp. *hordei*	242 (184)	6	17	Unknown	Xylem	Suppression of I-2 and I-3 resistance	*I or I-1*	(Houterman, et al. 2007) (Stergiopoulos and de Wit 2009)
ATR1$^{Nd}_{WsB}$	*H. arabidopsidis*	311 (296)	-	15	induces HR, RXLR domain	cytoplasm	Suppress host defense	*RPP1*^{WsB} and *RPP1*Nd	(Rehmany, et al. 2005, (Sohn, et al. 2007)
ATR13	*H. arabidopsidis*	187 (168)	-	19	induces HR, RXLR domain	cytoplasm	Suppress host defense	*RPP13*	(Allen, et al. 2004) (Sohn, et al. 2007)
Avr1-b1	*P. sojae*	138 (117)		21	induces HR, suppress BAX induced cell death, RXLR domain	Probably cytoplasm	Unknown	*Rps1b and RpsK1*	(Shan, et al. 2004) (Dou, et al. 2008a)
Avr1a	*P. sojae*				RXLR domain		Unknown	*Rps1a*	(Qutob, et al. 2009)
Avr3a	*P. sojae*				RXLR domain		Unknown	*Rps3a*	(Qutob, et al. 2009)
Avr3c	*P. sojae*	220			induces HR, RXLR domain		Unknown	*Rps3c*	(Dong, et al. 2009)

Protein	Organism	Length aa residues (mature)	No: of Cysteines	Signal Peptide in aa	Biological activity/homology	Protein localization	Role in virulence/pathogenicity	Corresponding R-gene	References
Avr3a	*P. infestans*	147		21	induces HR, suppress INF1 induced HR RXLR domain, interact with CMPG1 ubiquitin E3 ligase	cytoplasm	Required for full virulence	*R3a*	(Armstrong, et al. 2005) (Bos, et al. 2006) (Bos, et al. 2009) (Bos, et al. 2010)
MfAvr4	*M. fijiensis*	121 (100)	10	21	Chitin-binding peritrophin-A, induces HR or necrosis in Cf4 toamto,	Probably in apoplast	Protect fungi against chitinases		(Stergiopoulos, et al. 2010)
MfEcp2	*M. fijiensis*	161(142)	4	19	induces HR or necrosis in CfEcp2 toamto, Unknown	Probably in apoplast	Promote virulence by interacting with host cell target causing necrosis		(Stergiopoulos, et al. 2010)

CHAPTER 2

The avirulence gene *UhAvr1* clusters with predicted secreted proteins in the *U. hordei* genome and is inactivated by transposon activity in virulent strains[1]

2.1. Introduction

Plants use a variety of defense mechanisms to avoid pathogen invasion and subsequent disease. These defense mechanisms include physical barriers, preformed antimicrobial compounds, and activation of induced defenses. As discussed in Chapter 1, triggers for such defenses can be highly conserved molecules from pathogens (Pathogens or Microbe Associated Molecular Pattern or PAMPs or MAMPs) resulting in PAMP-triggered immunity (PTI). On the other hand, pathogens secrete effector molecules into the host plant to avoid or suppress PTI and help in nutrient acquisition for pathogen growth and development (Kamoun 2007, van der Does and Rep 2007). Plants use a highly sophisticated system encoded by resistance (*R*) genes to recognize these effectors and trigger a variety of defense mechanisms, including a localized cell death called the hypersensitive response (HR) to arrest pathogen development (Keen 1990, Van der Biezen and Jones 1998). The pathogen molecules that are recognized by *R* genes are called avirulence (*Avr*) genes, since they render the pathogen unable to cause disease on that particular host. This type of resistance in the host and avirulence in the pathogen is called a gene-for-gene interaction (Flor 1942).

Pathogen effectors are rapidly evolving and, in many cases, avirulence genes are present in regions displaying high genome flexibility, such as telomeres (Orbach, et al. 2000), or heterochromatic locations (Fudal, et al. 2007, Parlange, et al. 2009), or they are surrounded by transposable elements (Fudal, et al. 2007, Gout, et al. 2006, Khang, et al. 2008, Zhou, et al. 2007).

[1]A version of the chapter entitled "The avirulence gene *UhAvr1* clusters with predicted secreted proteins in the *U. hordei* genome and is inactivated by transposon activity in virulent strains", is in preparation for publication. Anticipated co-author list: Ali, S., Linning, R., Laurie, J. and Bakkeren, G.

Pathogen Avr proteins are recognized by plant R proteins either directly through the receptor-ligand model or indirectly by changes in the host protein (Keen 1990, Van der Biezen and Jones 1998). Direct interaction results in diversifying selection of both *R* and *Avr* genes and an arms race between the pathogen and the host plant (Stahl and Bishop 2000). In indirect recognition systems, the R protein recognizes the pathogen's effectors through detection of changes in the host proteins that the effectors target, also called the guardees (Van der Biezen and Jones 1998). This model is called the "guard model" and seems to be a more common system than the receptor-ligand model.

The smuts are important pathogens that cause disease world-wide (Menzies, et al. 1996, Thomas 1989). In our laboratory, we are working on two closely related smuts, *U. hordei* and *U. maydis*, that infect barley and corn, respectively. *U. maydis* is a widely-studied pathogen and the complete genome for this pathogen has been sequenced, although no *Avr* genes have been described (Kamper, et al. 2006). The barley/*U. hordei* pathosystem is an excellent model system for small grain-infecting basidiomycetes due to the presence of "gene-for-gene"-based resistance. Six *Avr* genes have been described in *U. hordei* which in different combinations constitute 14 different reported races (Tapke 1945); six corresponding resistance genes have been identified in barley (Thomas 1976). The *UhAvr1* gene has been identified in a genomic region of 85 kb (Linning, et al. 2004). To our knowledge, no *Avr* gene from basidiomycetes infecting monocots has been isolated so far. In this study, I present the identification of the *UhAvr1 gene* from *U. hordei* by targeted deletions of clusters of predicted secreted proteins, located between genetic markers that were previously identified (Linning, et al. 2004). I also used a complementation-based approach to restore the avirulence function in virulent deletion mutants. The region containing the *UhAvr1* gene is syntenic to cluster 19A of *U. maydis* which contains small secreted proteins that have virulence functions (Kamper, et al. 2006). The data show that *UhAvr1* is located in a transposon- and repeat-rich region and that (retro) transposon activity is responsible for breaking the avirulence towards Hannchen *(Ruh1)*. In the virulent parent, it appears that this gene has been separated from its promoter and a region of more than 14 kb has translocated to another part of the genome.

2.2. Material and methods

2.2.1. Barley cultivars and *U. hordei* strains used in this study

Four barley cultivars, namely universal susceptible Odessa (*ruh1, ruh2, ruh6*), and differentials Hannchen (*Ruh1*), Excelsior (*Ruh2*), and Plush (*Ruh6*) were used for pathogenicity assays of *U. hordei* in this study. Table 2.1 lists all wild-type and deletion mutant *U. hordei* strains used and generated in this study.

2.2.2. Growth conditions of *U. hordei* and barley and *U. hordei* transformation

All haploid strains of *U. hordei* (Table 2.1) were grown in liquid complete medium (CM; Holliday et al. 1961) always supplemented with Ampicillin 100 μg ml^{-1} (Fisher Scientific), while Carboxin 2.5 μg ml^{-1} (Sigma-Aldrich), Hygromycin B 100 μg ml^{-1} (Calbiochem, La Jolla CA, USA) or Zeocin 40 μg ml^{-1} (Invitrogen, Valencia, CA, USA), were added when appropriate. Strains were grown for two to three days at 22 ^{o}C and then preserved in cryovials at -80 ^{o}C after adding 9% v/v filter-sterilized DMSO. When needed, strains were recovered on solid potato dextrose agar (PDA; potato starch 0.4%, dextrose 2%, agar 1.5%), CM or YEPS (1% yeast extract, 2% peptone, 2% sucrose, 2% agar for solid media) media incubated at 22 ^{o}C for 3 days. For genetic transformation of *U. hordei*, protoplasts were prepared according to a modified protocol (Tsukuda, et al. 1988) but using 384 mg ml^{-1} Vinoflow FCE (Gusmer Enterprises) as enzymes for dissolving the fungal cell wall (Szewczyk, et al. 2006). The prepared protoplasts were used either fresh or stored at -80 ^{o}C like haploid sporidia. The protoplasts were transformed with 5 μg DNA mixed with 1 μl of 15 mg ml^{-1} heparin (Sigma H-3125) in STC (10 mM Tris-HCL pH. 7.5, 100 mM CaCl2, 1M sorbitol) and selected on double-complete medium plate (DCM) supplemented with 1 M sorbitol and appropriate antibiotic. After 5-7 days incubation at 22 ^{o}C, colonies from DCM-S were transferred to CM medium and incubated for two days at 22 ^{o}C before transferring to liquid CM medium for further analysis.

Barley seeds were planted in general potting mix (Pro-Mix BX) in pots of 3 X 3 inches that were placed in trays to a density of 3 seeds per pot and 18 pots per tray; both pots and trays had small holes for water drainage. Trays were placed in controlled-environment chambers

(Conviron, Winnipeg Manitoba Canada) or in the green house with an 18 hour light-6 hour dark cycle and held at 22°C.

2.2.3. Mapping of the *UhAvr1*

The *UhAvr1* gene was mapped (Linning, et al. 2004) using a marker-based approach in a population of *U. hordei* haploid strains, Uh4857-4 (alias Uh364), Uh 4857-5 (alias Uh365), Uh4854-10 (alias Uh362), Uh4854-4 (alias Uh359) and their progeny. Briefly, a population was created by crossing Uh364 and Uh362 haploid strains on universal susceptible barley cultivar Odessa and fifty-four random progeny were selected, half had mating type *MAT-1* and half *MAT-2*. To identify virulence genotype, all the progeny were subsequently backcrossed to virulent parents (Uh362 or Uh359) depending on mating type, and tested for pathogenicity on differential cultivar Hannchen (*Ruh1*) and on Odessa (*ruh1*) as an inoculation control. This created a mapping population for measuring recombination frequencies. Bulked-segregant analysis was used for mapping *Uhavr1* in pools of eight progeny segregating for *UhAvr1* or *uhavr1*. BAC clone 3-A2 was isolated from the BAC library of the avirulent parent (Uh364) that contains the entire 85 kb *UhAvr1* locus. Subsequently, two overlapping BAC clones 1-E2 and 2-G7 were isolated from the virulent parent that span the *Uhavr1* locus.

2.2.4. Sequencing of ORFs in the *UhAvr1* locus from the virulent parent and field isolates

All open reading frames (ORFs) that coded for proteins predicted to be secreted and located in the *UhAvr1* locus between the genetic markers, were amplified by PCR (using a mixture of Pfu and Taq polymerase) from strain Uh362 (*avr1*) virulent on barley cultivar Hannchen, and field isolates. Primers for these ORFs were designed 100 bp upstream and 100 bp downstream of the ORFs (Table 2.2) using the Primer3 software. The PCR products were run on 1% agarose gels and purified using a QIAquick Gel extraction kit (QIAGEN) following the manufacturer's instruction. Sequencing of the purified products was carried out either at PARC (Summerland, BC) using the Big Dye terminator mix from Applied Biosystems and an ABI310 Genetic Analyzer (Foster City, CA, USA), or at the UBC-Okanagan campus in Kelowna, BC. The sequence data were compared using the sequence analysis and alignment software of the VectorNTI software package (Invitrogen). When appropriate, data were compared to the genome

sequence from *U. hordei* strain 364 *(MAT-1)* at MIPS (http://mips.helmholtzmuenchen.de/genre/proj/MUHDB/, to be made public in 2011) and from *U. maydis* strain 521 *(a1 b1)* at MIPS (http://mips.helmholtz-muenchen.de/genre/proj/ustilago/).

2.2.5. Sequencing of BAC clones from the avirulent and virulent parents by 454 method

The *UhAvr1* region of the avirulent parent (strain Uh364, represented by BAC clone 3-A2) had been sequenced using the GPS-Mutagenesis System (New England Biolabs) by J. Laurie (UBC Thesis, 2008; and unpublished). These sequences and those for this region obtained from the BAC end-sequencing of the source BAC genomic library (Genome Sciences Center, Vancouver, BC; unpublished data) were assembled using the PCAP.REP software suite (Huang, et al. 2006). Some manual sequencing was done to confirm regions and fill gaps. Genes were predicted using VectorNTI (Invitrogen) and FGENESH (Salamov and Solovyev 2000). Two BAC clones (1-E2 and 2-G7) were also obtained for the *Uhavr1* region from the virulent parent (strain Uh362) via hybridization, by J. Laurie (UBC Thesis, 2008; and unpublished). These two, as well as the BAC3-A2 clone from the avirulent parent were sequenced using the 454 technology at the Plant Biotechnology Institute (Saskatoon, Sask.) and the resulting reads assembled using the Newbler program (Roche Applied Science). Alignment of the BAC sequences from the virulent parent along the avirulent backbone was facilitated by a custom Perl script provided by Matthew Links (Agriculture Canada, Saskatoon, Sask.). This was again followed by confirmatory manual sequencing and PCR reactions.

2.2.6. Deletion of the *UhAvr1*-containing region

To make deletion mutants of the *UhAvr1* gene or clusters of genes in the *UhAvr1* locus, several plasmids were constructed using the DelsGate method (Garcia-Pedrajas, et al. 2010). Briefly, primers were designed separately for each construct to amplify 1.5-2 kb, 5′- and 3′- sequences flanking the target region (Table 2.2) by PCR using the Uh364 genomic DNA as a template. Primers 5L and 5R were then used for the amplification of the 5′ -flanking fragment which contains an I-SceI recognition sequence tail upstream and an *attB1* sequence tail downstream. Primers 3L and 3R were used to amplify the 3′-flanking fragment which then contained the *attB2* sequence upstream and the I-SceI sequence tail downstream. The two PCR-amplified fragments

were then gel-purified using the QIAquick Gel extraction kit and subsequently recombined into the pDonorCbx vector (NCBI accession number EU360889; Garcia-Pedrajas, et al. 2010) using the Gateway BP Clonase II enzyme Mix (Invitrogen). Two PCR reactions were performed for the verification of the deletion construct using 5′- gene-specific primer 5R in combination with the SceIF primer (Table 2.2), and 3′- gene-specific primer 3L in combination with primer SceIR primer (Table 2.2). SceIF and SceIR primers were designed for the I-SceI enzyme recognition site in the forward and reverse orientation, respectively. All deletion constructs used in this study were verified by sequencing. The deletion constructs were then linearized with I-SceI enzyme (New England, Biolabs), ethanol precipitated, and subsequently resuspended in STC (section 2.2.2) and used directly for transformation of *U. hordei* strains as described, above. Putative transformants (resistant to carboxin) on DCM-S media were transferred to CM media plates supplemented with carboxin for further analysis.

2.2.7. Analysis of deletion mutants

Carboxin-resistant mutants were analyzed for proper gene deletion by two PCR reactions amplifying the 5′- flanking part that was used for making the deletion construct with primer DonF sits on the construct and another primer 150-200 bp upstream of 5′-flanking gene in the genome (Table 2.2). The second PCR reaction was carried out to verify the 3′-flanking part using primer DonR from the construct and another primer 150-200 bp downstream of the 3′ flanking part in the genome. *U. hordei* deletion mutants that were positive for both PCR reactions were further verified by Southern bloting analysis to confirm proper homologous deletion of the gene. Southern blot analysis was carried out in the same way as described in Chapter 4 of this thesis. Briefly, genomic DNA from either PCR-positive deletion mutants or wild type strains was digested with two different restriction enzymes. A probe for either the 5′- or 3′- flanks was amplified using PCR in a way that would yield different size bands in the wild-type strain and deletion mutants.

2.2.8. Plasmids to complement *U. hordei* deletion mutants

ORFs of *Uh10021, Uh10022*, and *Uh10024* of *U. hordei* and ORFs *Um05295* and *Um05296* of *U. maydis*, either with or without the sequence coding for the signal peptide (SP), but without

their stop codon, were each amplified by PCR with a CACC tetranucleotide sequence at the 5′ end to allow for directional cloning into Gateway entry vector pENTR™ /D-TOPO™ (Invitrogen). Kanamycin-resistant colonies were verified by PCR and inserts were sequenced using primers M13F and M13R. Plasmid DNA was purified from *E. coli* using the plasmid mini extraction kit (QIAGEN) following the manufacturer's instruction. The genes were subsequently transferred to the expression vector, pUBleX1Int:GateWayHA (Fig. 2.1), using the LR recombineering reaction (Invitrogen). For the construction of pUBleX1Int:GateWayHA, a synthetic linker containing a HA epitope tag was used (bold, translates into YPYDVPDYA) and a stop codon (red), flanked by BamHI/BglII and KpnI cohesive ends (underlined) and an NruI restriction enzyme site (italic) for blunt-end cloning of GateWay™ recombineering cassette, reading frame B (to allow in-frame fusions with the HA epitope tag). Primers that were annealed to obtain this linker were: Bam-HAtag-Kpn_fw GATCC*TCGCGA***TATCCGTACGACGTACCAGACTACGCATGA**GGTAC and Bam-HAtag-Kpn_rev C**TCA**TGCGTAGTCTGGTACGTCGTACGGATATCGCGAG. The annealed product was ligated to a shuttle vector, cut with BamHI and KpnI. The GateWay™ cassette, reading frame B (Invitrogen), was inserted into the unique Nru1 site. Subsequently, the GateWay-HAtag-STOP cassette was amplified by PCR with primer Gateway-5'_BglII (GGAAGATCTCGATCAACAAGTTTGTAC) which adds a BglII site (underlined) and primer MCG161_nos_fw (agaccggcaacaggattcaatc) which sits upstream of another flanking BglII site in the shuttle vector. Because there is a third BglII site in the GateWay™ cassette, reading frame B fragment, the PCR products was digested partially with BglII to yield a 1,860 bp fragment which was inserted into the unique BglII site of integrative, *Ustilago*-specific expression vector pUBleX1Int (Hu, et al. 2007).

To complement *U. hordei* deletion mutants with a whole-gene construct, Uh10022 with its native promoter and terminator sequences was amplified by PCR using primers 1616 and 1617, each containing a NotI restriction enzyme site. The PCR product was digested with NotI and cloned in the NotI site of plasmid pHyg101 (Mayorga and Gold 1998) using T4 DNA ligase (Invitrogen). The construct was verified by PCR and linearized by SspI to allow stable integration in the genome of *U. hordei* following the transformation method described, above.

2.2.9. Western blot analysis

Total protein was isolated from frozen ground cells, as described (Laurie, et al. 2008). Protein samples were boiled for five minutes and spun briefly for 30 sec before being applied on a 12.5% SDS-PAGE for separation using a Bio-Rad Mini-Protean III. Protein was transferred from the gel to Eeqi-Blot PVDF Western blotting membrane (Bio-Rad) using a Bio-Rad liquid transfer apparatus following the manufacturer's recommended protocols. Western blot hybridizations were carried out according to a standard protocol (Harlow and Lane, 1988). Membranes were probed with 200 ng ml^{-1} rat anti-HA high affinity monoclonal antibody. For detection of primary bound antibody, membranes were incubated with peroxidase-conjugated AffiniPure Goat Anti-Rat-Ig (H+L) secondary antibody according to supplier's instruction. For visualization of bound antibody, the Enhanced Chemiluminescence system (ECL) plus Western Blotting Detection Reagents (Amersham Biosciences/GE Healthcare) were used.

2.2.10. Mating test

U. hordei mating tests were carried out for all deletion mutants before pathogenicity tests were performed on barley cultivars. Strains of interest were grown in CM liquid medium with appropriate antibiotics for 36-48 hours in a shaking incubator at 22 ^{o}C to reach an OD600 of 0.6-0.8. Two haploid strains of opposite mating type were then mixed 1:1 (v/v) and 30-50 µl were spotted on CM media plates supplemented with 1% (w/v) activated charcoal and incubated at 22 ^{o}C for 36-48 hours. A positive reaction in a colony, indicating mating, was visible as a white "fuzzy" phenotype.

2.2.11. Pathogenicity assays

Pathogenicity assays were performed as follows: Haploid *U. hordei* strains of opposite mating-type backgrounds (such as deletion mutant strains in the Uh364 (*MAT-1, Avr1*) to the Uh362 (*MAT-2, avr1*) parent) were grown separately in CM media with appropriate antibiotics in a shaking incubator for 36-48 hours to reach an OD600 of 1-1.5. The two cultures of opposite mating types were mixed 1:1 v/v before inoculation of the barley seeds. The deletion mutants were also mixed with Uh365 *(MAT-2, Avr1*) wild-type strains to complement the deletion mutant. Wild-type combination Uh364 x Uh362 was used as a control. Seeds of barley cultivar

Hannchen (*Ruh1*) and Odessa (*ruh1*) that were previously surface sterilized and dried were dipped in the mixed cultures and a vacuum of 20 psi was applied for 20 minutes. Subsequently, the seeds were placed in sterilized plates on tissues to drain the excess culture at room temperatures for 4-6 hours. The seeds were then sown in potting mix (Pro-Mix BX) as described, above. Disease rating was scored 2 months after heading of the plants by counting infected plants in all inoculated plants. All pathogenicity tests were repeated at least three times.

2.2.12. Nucleic acid manipulation

For deletion mutant analysis, total genomic DNA was isolated according to a modified protocol for miniprep of *Ustilago* genomic DNA (Elder, et al. 1983). For cloning and DNA blot analysis, total genomic DNA was isolated using the DNeasy Plant Maxi kit (QIAGEN Mississauga, Ontario, Canada) following the manufacturer's instructions (for more detail, see Material & Methods section 4.2.4 of genomic DNA isoalation in Chapter 4). Routine Polymerase Chain Reaction (PCR) was conducted using recombinant Taq polymerase (Invitrogen) or when required, Pfu polymerase (Fermentas Life Science). For cloning and labeling, PCR products were either gel-purified using a QIAquick Gel extraction kit (QIAGEN) or they were directly purified using the QIAquick PCR purification Kit (QIAGEN) according to manufacturer's instruction.

2.2.13. qRT-PCR analysis

Mated *U. hordei* wild-type strains Uh362 and Uh364 were inoculated on three day-old seedlings of barley cultivars Odessa and Hannchen. Inoculum was prepared by collecting cells from two day-old cell cultures by centrifugation, mixing of opposite mating types and then painting the cell paste onto coleoptiles with cotton swabs. Barley seeds were also inoculated in the same way as described, above, in section 2.2.11, and then germinated in the dark on moist filter paper in Petri plates. Samples were collected from inoculated plants at 24, 48, 72, 96, and 120 hours post inoculation and used for RNA isolation. RNA was also isolated from infected immature barley heads dissected from approximately 5 week-old plants and from mature heads filled with teliospores. As a control, RNA was isolated from *U. hordei* cells mated on charcoal plates. 1 μg of total RNA that was isolated and purified using a QIAquick RNeasy extraction kit (QIAGEN)

according to the manufacturer's instructions, was treated with DNaseI, amplification grade (Invitrogen). First-strand cDNA was synthesized using a Dynamo SYBR Green 2-step qRT-PCR kit (FINNZYMES) following the manufacturer's instruction. These samples were then diluted ten times and real-time qPCR was performed with specific primers on an Mx3000P qPCR instrument (Stratagene, La Jolla, CA, USA). Amplification cycles were as follows: 15 min at 95 ^{o}C, followed by 40 cycles of 30 s at 94 ^{o}C, 30 s at 63 ^{o}C, 30 s at 72 ^{o}C; fluorescence data were collected at 63 ^{o}C at each cycle. The reliability of the product was verified by acquisition of a dissociation curve at the end of each run. The sequence of the primers for the genes of interest and the housekeeping control gene are shown in Table 2.2.

2.3. Results

2.3.1. Sequencing of ORFs from the virulent parent and field isolates

Lining, et al. (2004) identified three genetic markers linked to *UhAvr1* in a mapping population of 54 progeny segregating for this gene (Fig 2.2). The AFLP marker was converted to a probe for screening a cosmid library. Part of a positive cosmid clone was then used as a probe to identify from a BAC library from avirulent parent, Uh364 (*Avr1*), five overlapping BAC clones that spanned the whole locus (Lining R 2004). A single BAC clone, BAC3A-2 was selected because it contained the entire *UhAvr1* locus. This clone was sequenced by GPS transposon insertion resulting in a sequence of 117 kb with two small gaps (J. Laurie, UBC Thesis, 2008; and unpublished). Genes were predicted using VectorNTI (Invitrogen) and FGENESH (Salamov and Solovyev 2000) and in this region 47 ORFs were identified (Table 2.3). The sequence analysis of this BAC clone revealed that *UhAvr1* locus is syntenic to a region in *U. maydis* on contig 1.191 spanning a complete cluster, called 19A, the largest cluster in *U. maydis* harbouring 26 small secreted proteins (Kamper, et al. 2006).

To identify the *UhAvr1* gene by sequence comparison, I hypothesized that it could encode a secreted protein. Predicted secretion signals in eight ORFs were identified by SignalP 3.0 and TargetP 1.1 in the region identified by genetic marker analysis; three fall outside this region (Fig 2.2, RAPD and RFLP; Lining et al., 2004). A change from avirulence to virulence (or vice versa) could potentially be caused by mutations in the candidate gene; therefore, I wanted to compare candidate genes encoding putative secreted proteins between the avirulent

and virulent parent, and among a collection of eight field isolates collected from different parts of the world whose virulence was known (Table 2.1; two avirulent and six virulent on barley cultivar Hannchen, *Ruh1*). These predicted ORFs with secretion signals were sequenced from virulent strain Uh362 (*avr1*) and the eight field isolates. ORFs were amplified by PCR from genomic DNA with primers designed 100 bp upstream and 100 bp downstream of each ORF (Table 2.2). The PCR products were sequenced directly using the primers listed in table 2.2. Sequence analysis revealed point mutations in four ORFs, *Uh10021, Uh08127, Uh08128* and *Uh08132*, between the parental strains. In *Uh10021*, two point mutations were identified, one at 21 nt upstream of the ATG start codon and the other at 165 bp downstream of the ATG. The latter mutation, G-to-A, changes a valine to an isoleucine in the virulent parent. *Uh08127* had one point mutation in the ORF, 657 bp downstream of the start codon, an A-to-G that translates into a single amino acid difference between the parental strains, a methionine into an isoleucine in the virulent parent. One point mutation was identified in *Uh08128*, 264 bp downstream of the ATG, a T-to-C translating a serine into a proline in the virulent parental strain. In gene *Uh08132*, a single point mutation in the stop codon shifts the frame, resulting in a longer protein sequence in the virulent parent. Among the field isolates, only one, Uh813 collected from Iran, revealed a point mutation in the ORFs of *Uh08127, Uh08128, Uh08132, Uh08139* and two bp changes in *Uh10022,* that each translated into single amino acid changes. Unfortunately, all these above mentioned point mutations did not correlate with the genotypes *Avr1* or *avr1* in the field isolate collection and were, therefore, not followed further for avirulence gene analysis.

2.3.2. Delimiting of the *UhAvr1*-containing region by deletion analysis

To identify the *UhAvr1* gene in the 85 kb region between the markers, it was divided into four overlapping fragments (Fig 2.3), ranging from 15 to 38.5 kb based on the number of ORFs in the region coding for predicted secreted proteins. The DelsGate method (Garcia-Pedrajas, et al. 2010) was used to prepare the deletion mutants of these fragments (see Material and Methods). The deletion constructs were then transferred to Uh364 (*Avr1*) protoplasts to make the *Avr1* deletion mutants. Sixty to as many as 300 carboxin-resistant colonies sometimes needed to be screened to get at least four PCR positive transformants for each construct. These putative deletion mutants were further verified by Southern blot analyses (Fig. 2.3) to confirm expected homologous deletion mutants. The efficiency of homologous recombination was different for

different constructs and seemed dependent on the size of the deletion fragment; the efficiency was higher for small fragments. No phenotypic differences or abnormal growth were observed for any of the tested haploid basidiospore mutants and, also, proper mating with compatible haploid basidiospores was observed. At least one deletion mutant for each fragment was inoculated on barley cultivars Hannchen and Odessa after mixing with compatible virulent parent Uh362. The deletion mutants that were used for pathogenicity tests are listed in Table 2.1. Inoculation of deletion mutants and the wild-type *U. hordei* strains on barley cultivars Odessa *(ruh1)* and Hannchen *(Ruh1)* clearly indicated that the fragment called C19A2 contained the avirulence gene *Avr1* (Fig 2.4). The wild-type strain Uh364 and the mutants deleted for three fragments, C19A3, C19A4 and C19A5, when mated with virulent partner Uh362 of the opposite matting type, caused disease on Odessa but not on Hannchen, indicating that these deletion mutants were avirulent on barley cultivar Hannchen and, therefore, still contain a functional *Avr1*. The C19A2 deletion mutants however, caused disease on both barley cultivars when mated with virulent parent Uh362, proving that the *UhAvr1* gene is present on this fragment. This deletion mutant strain was named Uh1041 (Uh364-Δ*19A2* Table 2.1) and will be referred to as such for the remainder of the thesis. Strain Uh1041 also causes a higher rate of disease towards Odessa than the wild-type strains (Fig 2.4). To independently test whether the disease on barley cultivar Hannchen was the result of *UhAvr1* deletion, I also inoculated this barley cultivar with a cross of Uh1041 with the avirulent strain Uh365 (*Avr1*), which resulted in disease on Odessa but not on Hannchen. I also used another control in which Uh1041 (Δ*Avr1*, *Avr6, Avr2*) was inoculated on two other barley differential cultivars, Plush and Excelsior, that have the resistance gene, *Ruh6* or *Ruh2*, respectively, after crossing with Uh362 (*avr6, avr2*). Uh1041 did not produce disease on any of these cultivars, which verifies that the virulence of this mutant on Hannchen is due to deletion of the *UhAvr1* gene in this region.

2.3.3. Deletion of fragment C19A2 in both mating partners does not impair virulence towards Odessa

In other pathosystems, the deletion of avirulence genes/effectors was shown to affect virulence. To determine whether genes on fragment C19A2, which includes 5 SSPs, have any virulence functions in *U. hordei*, I needed to construct a deletion mutant in the mating partner as well. A mutant deleted for the C19A2 fragment was obtained in the other mating type partner by

crossing Uh1041 (C19A2 deletion mutant, *MAT-1*) with the virulent parent, Uh362 (*MAT-2*), on barley cultivar Hannchen. Basidiospores were collected from teliospores of infected heads and selected for carboxin resistance. Carboxin-resistant basidiospores of mating type 2 (*MAT-2*) were obtained by performing mating tests with Uh364 (*MAT-1*) and Uh359 (*MAT-1*) on DCM plates supplemented with active charcoal. Deletion mutants of mating type 2 were further verified by Southern blot analysis (Fig. 2.5). Seeds of barley cultivar Odessa and Hannchen were then inoculated after mixing equal amounts of Uh1041 culture with C19A2-deletion mutants of mating type 2, and disease was scored after heading of the barley plants.

Three individual C19A2 deletion mutants, Uh1116, Uh1117 and Uh1118, of mating type 2 were tested in this study. Virulence of the cross lacking any C19A2 components towards Odessa was similar to the wild-type cross (Fig. 2.5), as measured by counting percent diseased plants out of total inoculated plants. The percentage disease on Hannchen seemed also not affected compared to the single deletion mutant, indicating that the genes located on fragment C19A2 did not contribute significantly to virulence on barley.

2.3.4. Complementation of C19A2 deletion mutants

A 38.5 kb fragment, C19A2, contains the functional *UhAvr1* gene; this fragment encodes five predicted secreted proteins, three of which are located between the genetic markers delineating the locus. I focused on these three genes as potential candidates for *UhAvr1*. A BAC subclone was identified in this region that contains two of the predicted secreted protein-coding ORFs, *Uh10021* and *Uh10022* (Fig 2.6). BAC 1-6 was previously cloned in pUSBAC5 (BAC vector derivative of pEcBAC1; (Frijters, et al. 1997). and converted for use in *Ustilago* species by introducing a specific hygromycin B resistance cassette (Linning, et al. 2004). Two C19A2 deletion mutants complemented with BAC1-6 (Uh1205 and Uh1207; Table 2.1) were inoculated on barley cultivars Odessa and Hannchen after mixing with the compatible virulent strain. No abnormal growth or defect in mating behavior was observed in these haploid complemented strains. The complemented strains caused the same level of disease on Odessa as the wild-type combination and the deletion mutants, while on Hannchen the level of disease was severely reduced (Fig 2.6). On Odessa, the disease level varied from 25-40% while on Hannchen the disease was only 2-2.5%, which strongly suggests that BAC 1-6 contains the functional *UhAvr1* gene.

To find the *UhAvr1* gene, the deletion mutant strain Uh1041 was individually complemented with each of the three genes, *Uh10021, Uh10022* and *Uh10024*. For complementation with the individual genes, these ORFs were cloned both with signal peptide (predicted by SignalP 3.0) and without signal peptide in integrative plasmid pUblexInt:GateWayHA under control of the HSP70 constitutive promoter. Deletion mutant strains complemented with these different genes were analyzed by Western blot analysis to confirm the expression of the transgenes. As shown in Fig 2.7, all genes were expressed at a high level in all of the complemented lines tested. Two complemented strains for each individual gene were selected for pathogenicity tests on the barley cultivars, Odessa and Hannchen. The pathogenicity data did not confirm that these individual genes are able to complement the avirulence function in the 38.5 kb deletion mutant strain, Uh1041 (Fig. 2.7).

2.3.5. Fragments C19A2-C and C19A2-D contain *UhAvr1*

In order to determine the location of the *UhAvr1* gene on the 19A2 fragment of 38.5 kb, this region was divided into five sub-fragments, C19A2A-C19A2E (Fig 2.7), for making further deletion mutants. To generate the sub-deletion mutant constructs, the primers were designed in such a way that the three predicted secreted protein encoding ORFs would be deleted in two different deletion constructs (Fig 2.8). The 1.5 to 2 kb flanking regions of the targeted fragments were amplified by PCR using the primer combinations listed in Table 2.2 and subsequently cloned in pDONR-Cbx by BP clonase II. The sub-deletion constructs were then linearized with I-SceI enzyme for integration into the genome of Uh364. Sixty-four PCR-positive deletion mutants were obtained for the five deletion constructs, which were further verified by Southern blot analysis (Fig 2.8). Nine deletion mutants were selected, two for each deletion mutant, except for C19A2-B for which only one expected deletion mutant was obtained, and were tested for virulence towards Hannchen. The virulence of each of these deletion mutants after mating with the virulent partner Uh362 (*avr1*) on Hannchen was assessed the same way as described, above. Four deletion mutants, two for C19A2-C and two for C19A2-D, were virulent towards both barley cultivars Odessa and Hannchen (Fig 2.9). The virulence of these mutants towards Hannchen is because of disruption of the *UhAvr1* gene. The deletion mutants for the other three fragments, two for each of C19A2-A and C19A2-E, and one for C19A2-B, produced disease on Odessa, the universal susceptible host but not on Hannchen (*Ruh1*). These findings indicate that

each deletion mutant had the intact *UhAvr1* gene and, therefore, could not cause disease on Hannchen that recognizes *UhAvr1*.

2.3.6. Overlapping regions of the fragments C19A2-C and C19A2-D contain *UhAvr1*

As deletion mutants of both fragments, C19A2-C and C19A2-D, were virulent towards Hannchen, the overlapping region in these fragments was hypothesized to contain the functional *UhAvr1* gene. I found *Uh10022* as the only ORF in this region using VectorNTI (Invitrogen) and FGENESH (Salamov and Solovyev 2000). *Uh10022* encodes a predicted secreted protein (as identified by SignalP 3.0, TargetP 1.1 and ProtComP 9 prediction) and was a strong candidate for the *UhAvr1* gene. Another deletion mutant was produced in which the 3′-end (319 bp) of the *Uh10022* ORF was deleted by making a construct (Fig. 2.10) in which 2 kb flanking each side of this fragment was amplified by PCR using primer combinations listed in Table 2.2. The two amplified DNA fragments were cloned into pDONR-cbx as described, above, and linearized for integration into genomic DNA of the avirulent strain Uh364. A total of 110 carboxin-resistant colonies were screened by PCR for the proper deletion mutant and ten colonies were identified to be positive for both flanks. The Southern blot analysis of the eight transformants showed that five of them were proper homologous deletion mutants. Two of these transformants were selected and inoculated on the barley cultivars, Hannchen and Odessa. Both deletion mutants were virulent toward Hannchen and produced 30-40 % disease, which confirmed that *Uh10022* is the *UhAvr1* gene (Fig 2.10).

Quantitative RT-PCR was used to analyse the expression of *Uh10022* at various life cycle stages. RNA was isolated from plants infected with virulent and avirulent strains from mature or immature infected heads, or from *in vitro*-grown or mated basidiospores (see Material and Methods); however, the expression of this gene was not detected during infection under the conditions tested (data not shown). The expression of the housekeeping control gene was detected in the RNA samples collected from cells mated on charcoal plates, in the mated cell mix inoculated on the coleoptiles, and in both immature and mature infected heads; however, in all these conditions, expression of *Uh10022* was not detected. The expression of the housekeeping gene or *Uh10022* in RNA samples collected from inoculated seeds was not detected, likely because of limited fungal biomass (data not shown).

2.3.7. Sequence comparison between the *Avr1* and *avr1* loci in the parental strains

After genetically confirming that *Uh10022* is the *UhAvr1* gene, this gene was sequenced from BAC clone 1E-2 isolated from virulent parent Uh362 (*avr1*) using several primers combinations as listed in the Table 2.2. The homolog of *Uh10022* was previously obtained by PCR from genomic DNA of the virulent parent, Uh362, and sequenced; however, further analysis revealed that this gene was not present on BAC clone 1-E2, nor on BAC2-G7, although these two BAC clones are overlapping and span the whole region as shown in Figure 2.11. The sequence obtained from BAC1-E2 (Material and Methods) matched the sequence of the avirulent parent up to 134 bp upstream of the *Uh10022* start codon. After this point, which I called the break point, the sequence was no longer syntenous with the avirulent parent (Fig. 2.12). WUBLAST analysis of the 400 bp sequence after the break point indicated matches to two retrotransposon genes (Uh14086, Uh14170) in the *U. hordei* genome. PCR analysis of *Uh10022* and several other downstream genes on the BAC3-A2 clone revealed that almost 14 kb (containing nine predicted ORFs) was not present at this locus on BAC clones 2-G7 and 1-E2 from the virulent parent. PCR analysis confirmed the presence of all other ORFs flanking this 14 kb gap on these two BAC clones (Fig. 2.12). This was further verified by sequencing the ends of these two BAC clones and re-confirmed that they are overlapping and reside in this region. PCR was used on genomic DNA of the virulent parent, Uh362, for amplification across the break point using several primers combinations (Table 2.2), but no combination resulted in a PCR product. This suggested that these two regions are not physically close in the genome; however, as shown in section 2.3.1, I can amplify by PCR and sequence the ORFs of *Uh10021* and *Uh10022* from genomic DNA of the virulent parent but cannot amplify them as one physical fragment by PCR from the virulent parent, although both ORFs and the intergenic region span less than 2 kb in the avirulent parent. To measure the insertion, a PCR product of 3 kb was amplified from genomic DNA from the virulent parent using primer 1513 (600 bp downstream of the break point in the forward direction) and primer 1741 (within gene, Uh10026, in the reverse orientation; Table 2.2, Fig. 2.12). The combined data suggest that in the virulent parent, this 14 kb-region at this locus has translocated to another part of the genome and has been replaced by an insertion of 3.6 kb, harbouring transposable element (TE) sequences. It is likely that this event was caused by TE activity (Fig. 2.11). This translocation separated the *Uh10022* ORF from its promoter and likely

caused changes in the expression of the *Uh10022* (*UhAvr1*) gene and, hence, a conversion to a virulence genotype.

2.3.8. Variable sequences at the *UhAvr1* locus point to TE activity

The *Uhavr1* locus was sequenced from several virulent field isolates (Uh805, Uh815, Uh820, Uh822, Uh811 and Uh818) collected from different parts of the world (Table 2.1). PCR amplification across the break point failed in all virulent field isolates when several primer combinations matching different positions for the avirulent parent Uh364 were used (Table 2.2). To sequence the region spanning the break point, PCR products were generated using the primer combinations used for the sequencing of the region from the virulent parent BAC clone 1-E2. The sequence analysis of the PCR-amplified fragments confirmed the insertion of TE sequences (as identified in virulent parent Uh362) in all virulent field isolates sequenced in this study. This suggests that *Uh10021* and *Uh10022* are not physically connected to each other in the genome of all of these field isolates and is similar to the virulent parental strain Uh362; TE sequence insertions are present at this locus. Several mutations were revealed among the transposable element sequences in the different virulent strains. One predominant mutation found in four virulent strains (Uh362, Uh805, Uh815, and Uh820) is a ten bp-insertion of a repeat (GAGAGAGAGC) that is absent from three other virulent strains (Uh811, Uh818, and Uh822).

The *UhAvr1* locus was also sequenced from three avirulent field isolates (Uh813, Uh1273, and Uh1283) that were collected from different parts of the world (Table 2.2). In avirulent field isolate Uh813, a two bp mutation in the ORF of *Uh10022*, 506 bp downstream of the ATG, was identified that translates into a single amino acid change (isoleucine to argenine) in the C-terminal end of this protein. Since this strain is still avirulent on Hannchen, this change does not seem to affect its avirulence function. The locus from these field isolates was similar to the avirulent parent, Uh364. *Uh10021* and *Uh10022* are close to each other in the genome and could be amplified by PCR. Primers 13897F and 10022R (Table 2.2) were used for amplification of *Uh10021* and *Uh10022* on one PCR product but revealed that these products were roughly 340 bp larger in these strains than in the parental strain Uh364. Interestingly, in all the three avirulent field isolates tested here, the 340 bp addition was caused by an insertion in the intergenic region between *Uh10021* and *Uh10022* (Fig. 2.13). WUBLAST analysis of the 340 bp-insertion in these three avirulent strains indicated matches to TE sequences in *U. hordei*. The

340 bp-insertion was flanked by six bp repeats (TGGGTT), possibly a footprint of TE activity (Fig. 2.13). This insertion was not found in the virulent parent Uh362 or in the six virulent field isolates tested here. Overall, these sequence analyses and the presence of TE-related sequences suggests TE activity at this locus.

2.3.9. Lack of complementation of the C19A2 deletion mutant with *U. maydis* homologs of Uh10022

A search using WUBLAST of the Uh10022 protein sequence identified one paralog in the *U. hordei* genome (Uh10021) and three orthologs in the *U. maydis* genome (Um05295, Um05296/Um12302, and Um05294). Phylogenetic analysis of these protein sequences, including two more related *U. maydis* proteins, Um05297 and Um05298, revealed that Uh10022 is a homolog to Um05295 and Um05296 (Fig. 2.14). To determine whether the two *U. maydis* genes are functional homologs of *Uh10022*, both genes were cloned in the pUblexInt:GateWayHA expression vector expressed from the constitutive Hsp70 promoter, using primers listed in Table 2.2. Upon transformation, the expression of these genes in the complemented deletion mutant strains was similar to those complemented with *Uh10022* based on protein blot data (not shown). Four complemented *U. hordei* C19A2 deletion mutants, two for each of *Um05295* and *Um05296*, were selected for pathogenicity assays on barley cultivars Odessa and Hannchen. These complemented deletion mutant strains were crossed with virulent strain Uh362 before inoculation on barley seeds. All complemented strains were virulent on Hannchen and produced levels of disease on this cultivar similar to Uh1041 (Fig. 2.15).

2.3.10. Synteny between *U. hordei* and *U. maydis* at the *UhAvr1* locus

As mentioned above, the *UhAvr1* locus is syntenic to the region harbouring *U. maydis* cluster C19A, which contains twenty six predicted secreted protein encoding genes. In *U. maydis*, deletion of this cluster resulted in reduced disease on corn seedlings (Kamper, et al. 2006). SIMAP analysis and two-directional BLASTP searches were used to find orthologs of all proteins at this locus in the *U. maydis* genome. Orthologous proteins were found for several proteins at this locus (Table 2.3). The synteny is highly conserved on both flanks of cluster C19A (Fig 2.16); however, the sequences of the predicted secreted protein encoding ORFs are much diverged and rearrangements, including changes of gene orientation and several translocations of

genes within the cluster, are apparent. Genes encoding DigA protein on one side of the region are co-linear between the two species revealing proteins of very similar lengths. On the other side of the locus, two genes encoding a tubulin beta chain protein and another protein related to a VPS10 domain-containing receptor, SorCS1 precursor, are conserved, revealing a similar transcriptional orientation. In *U. maydis*, adjacent to the *digA* gene, an oligosaccharyltransferase gene is directly flanking the cluster of genes for secreted proteins, while in *U. hordei* this gene is transcriptionally inverted and located on the other side of the fragment that is shown by the red colored two-sided arrows (Fig. 2.16). This region is 38 kb larger than in *U. maydis*, seemingly the result of insertions of transposons and repetitive DNA (Fig. 2.16). In *U. maydis*, there are five families of SSP genes that are tandemly arranged in clusters of several paralogs. In *U. hordei*, most of these families are represented by a single gene; in two cases, the family is represented by two paralogs. *UhAvr1* shares little homology with two *U. maydis* genes however they are in strikingly syntenic locations. In contrast, in *U. hordei*, there are several transposons and repeats located at this locus interspersed with the SSP coding-genes. As shown by the two-sided arrows in Figure 2.16, two fragments of *U. maydis* containing several ORFs appear in opposite orientation in *U. hordei*. Also, both of these fragments in *U. hordei* are much larger than in *U. maydis*, possibly caused by insertion/activities of transposons, since three of these genes are related to TEs.

2.4. Discussion

The isolation and characterization of avirulence genes from different plant pathogens including fungi has been a long-term goal in plant pathology. Avirulence genes play key roles in determining genetic compatibility with plants and can induce resistance in host plants having corresponding resistance genes, the so-called effector-triggered immunity. I have shown in this study that *Uh10022* is the *UhAvr1* gene responsible for the induction of the defense response in Hannchen in a gene-for-gene manner. Avirulence towards *Ruh1* is broken down by TE activity and translocation of the coding region of *UhAvr1*, removing its promoter region and likely disrupting expression.

In this study, I aimed to identify the *UhAvr1* gene in *U. hordei* that was previously mapped to an 85 kb genetic interval through a genetic marker-based approach (Linning, et al.

2004). Eleven ORFs were identified at this locus that encodes predicted secreted proteins. They were annotated as 'hypothetical' with no known matches in public databases. To identify *UhAvr1* in the 85 kb region, I first used sequence comparisons of the predicted secreted protein coding ORFs between the two parental strains of the population and several field isolates collected from different parts of the world. The objective was to look for mutations linking genotypes *UhAvr1* or *Uhavr1* to phenotypes among the virulent and avirulent parental and field strains; however, the sequence comparisons of these ORFs did not provide conclusive data for the identification of a gene candidate for *UhAvr1,* despite several point mutations revealed in several ORFs between the virulent and avirulent parents (i.e., *Uh10021, Uh08127, Uh08128* and *Uh08132*). It is possible that some of the point mutations that I identified in several of the small secreted proteins could be related to some other avirulence or virulence genes in this pathogen not recognized by *Ruh1* in Hannchen. Nevertheless, the data presented demonstrate that the change from avirulence (*UhAvr1*) to virulence (*uhavr1*) is not due to the mutations in the ORFs or the presence or absence of ORFs in these two strains. The alternative hypothesis was that the avirulence gene may not be expressed in virulent strains because of a mutation(s) in the promoter region, possibly a promoter disruption by a transposable element insertion, as has been shown for several other avirulence genes from fungi (Kang, et al. 2001).

A targeted deletion and complementation-based approach was used to identify the *UhAvr1* gene and demonstrate that this gene is required for *Ruh1*-based resistance in barley cultivars towards *U. hordei*. A deletion mutant was produced by the DelsGate method (Garcia-Pedrajas, et al. 2010) that showed virulence towards barley cultivar Hannchen (*Ruh1*) while still avirulent to other cultivars such as Plush (*Ruh6*) and Excelsior (*Ruh2*). The deletion mutant Uh1041 in which a 38.5 kb region (C19A2) was deleted, could be complemented with 11 kb genomic fragment containing the predicted secreted proteins Uh10021 and Uh10022; however, this restoration of avirulence towards Hannchen was not complete and a very low level of disease remained that varied from 2-2.5% compared to the deletion mutant which produced 40% disease. I speculate that the reason for this low level of disease could be the result of reduced expression of transgenes not at the same level as in the wild type avirulent strain. Inadequate expression could result from two reasons; either the fragment, including the genes contained within it, being integrated into a part of the genome that expressed at a low level or the fragment was not complete and transgenes did not contain sufficient promoter sequences. I did not verify

the expression of the genes. Similar results have been shown for *Fusarium oxysporum* f. sp *lycopersici* mutant strains complemented with the *Six1* (*Avr1*) avirulence gene that did not restore complete avirulence towards tomato lines that contained the resistance gene *I-3* (Rep, et al. 2004).

To identify the gene that is specifically responsible for the avirulence phenotype in Hannchen, the C19A2 fragment was further divided into five deletion fragments. This result in the identification of a region overlapping in fragments C19A2-C and C19A2-D, pointing to *Uh10022* as *UhAvr1*. This was further confirmed by deleting 340 bp from the 3′-end of *Uh10022*; however, complementation of the C19A2 deletion mutant with *Uh10022*, both with and without the N-terminal signal peptide, was not successful in restoring the avirulence function towards Hannchen. The complementing gene in these integrative constructs was expressed from the constitutive Hsp70 promoter, causing over-expression, which may have interfered with proper processing and translocation into plant cells. Alternatively, it is possible that the transgenes were hampered in their activity because of position effects and may be functional only at their native avirulence gene's locus. Another scenario is that UhAVR1 needs another gene close by for its proper function, possibly required either for mRNA stability or proper folding of protein after translation. In the C19A2 deletion mutant, 38.5 kb is deleted that contains several genes in addition to *Uh10022*. In the oomycete *Phytophthora sojae* at least one avirulence gene *Avr1b-1* needs another gene *Avr1b-2* for mRNA accumulation and the avirulence function towards soybean (Shan, et al. 2004). The two orthologs of *Uh10022* from *U. maydis* also did not complement the avirulence function towards Hannchen. However, my experiments are not conclusive as to whether they are properly transferred and active in the host and are thus true functional homologs of *Uh10022*, similar to the lack of complementation as disussed for *Uh10022*.

One characteristic feature of *Avr* genes is that their expression is induced inside the plant during infection (Dodds, et al. 2004, Lauge and de Wit 1998, Rep, et al. 2004). I did not detect the expression of *Uh10022* in any of the conditions tested. As discussed earlier, after penetration, *U. hordei* proliferates heavily only in the developing head and seeds in inflorescences, where the fungus induces the disease symptoms. The RNA samples collected from germinating, inoculated seeds did not have detectable levels of expression even of the housekeeping gene, suggesting very low levels of fungal biomass at this stage. In all other samples, i.e. immature and mature

infected heads and mated cells from charcoal plates, high levels of expression were detected for the housekeeping gene but not of *Uh10022*. Based on these findings, I speculate that the expression of *Uh10022* is highly regulated and might be expressed only during the early stage of infection and possibly at a very low level. Some fungal *Avr* genes are expressed in specific infection structures such as appressoria or haustoria (Bohnert, et al. 2004, Dodds, et al. 2004, Catanzariti, et al. 2006). On the other hand, some fungal *Avr* genes are expressed during the whole infection process uniformly and are not organ-specific (Lauge and de Wit 1998, Luderer, et al. 2002b, Rohe, et al. 1995). Two *Avr* genes from *L. maculans, AvrLm1* and *AvrLm6*, have been shown to be expressed constitutively at low levels (Fudal, et al. 2007).

UhAvr1 is predicted to encode a small secreted protein of 171 aa after cleavage of a signal peptide with no cysteine residues in the mature protein. Such low content of cysteine residues has also been found for other fungal genes cloned by map-based strategies. Cysteine-poor effectors are common in biotrophic pathogenic fungi such as the basidiomycetes and oomycetes that form close associations with the host plants through haustoria (Allen, et al. 2004, Armstrong, et al. 2005, Ellis, et al. 2006, Rehmany, et al. 2005, Shan, et al. 2004). These secreted effectors are suggested to be translocated to the cytoplasm and, thus, spend only a short time in the extracellular space where they could be degraded by proteases. (Birch, et al. 2006, Dodds, et al. 2004, Dou, et al. 2008b, Haas, et al. 2009, Jiang, et al. 2008, Kale, et al. 2010, Khang, et al. 2010, Tyler, et al. 2006, Whisson, et al. 2007). Although *U. hordei* does not make haustoria within barley plant cells, it forms close interactions with interfaces surrounding mycelial tubes sometimes transversing plant cells within the host plant (Hu, et al. 2003). Similarly, putative secreted protein, such as AVRLm1 from *L. maculans*, has only one cysteine and does not form any disulphide bond; this fungus also does not form haustoria in its host (Gout, et al. 2006). In contrast, several fungal pathogens that colonize the plant apoplast encode avirulence proteins that are cysteine-rich and often have an even number of cysteine residues providing the means to form disulphide bridges (Rep, et al. 2004, Thomma, et al. 2005, van den Burg, et al. 2003, van den Hooven, et al. 2001, Stergiopoulos, et al 2010).

Several bacterial avirulence proteins have been shown to enter host cells and interact with a virulence target inside the cell, thus, playing a role in suppression of the host defense when the plant does not have the corresponding resistance protein (Chapter 1). The fact that no difference in disease is seen on Odessa when inoculated with *U. hordei* having avirulence gene *UhAvr1* or

the recessive allele *uhavr1*, suggests UhAVR1 is not significantly involved in virulence toward barley; however, this assessment was not done using isogenic strains. The C19A2 deletion mutant was created in the Uh364 (*MAT-1 Avr1*) parental strain and since this mutant Uh1041 became virulent towards Hannchen, it was deduced that it no longer contained a functional UhAVR1; therefore, a progeny of the other mating-type (*MAT-2*) was selected in which the C19A2 fragment was also deleted by crossing Uh1041 to parental line Uh362 (*MAT-2 avr1*). Inoculation of the new cross on barley cultivars Odessa and Hannchen revealed no significant difference in virulence towards these cultivars. This suggests that *UhAvr1* does not contribute significantly to virulence toward these barley cultivars. This may be explained by different scenarios. The first explanation may be that measuring infected plants out of the total number of inoculated plants is not a very sensitive assay for quantifying the virulence of pathogens towards their host. It is possible that they cause subtle variations that might become detectable in a population study. Second, these genes may have only additive effects on virulence. In *U. maydis*, some genes in cluster 19A also have additive effects on virulence towards maize seedlings (Brefort 2008). Therefore, this experiment needs to be reassessed in light of the later finding that in the virulent strain Uh362, genes 17 to 25 are translocated to another genomic location. Since it is not know yet whether this translocated fragment is on the same chromosome, when selecting for the C19A2 fragment deletion in the progeny with mating type *MAT-2* (using carboxin resistance), it cannot be sure these genes are no longer present in this progeny. I cannot deduce that genes 17 to 25 are not contributing to virulence; however, since gene number 17 (*UhAvr1*) no longer has a promoter and is not causing an avirulent phenotype in the virulent parent, it is likely not expressed. On these grounds, I conclude that UhAVR1 does not contribute significantly to virulence. We are currently verifying the location of the translocated fragment in virulent strains, but the experiment should be repeated in isogenic strains by crossing the generated *UhAvr1* 3′-deletion mutant with avirulent parental strain Uh365 (*MAT-2 Avr1*) and selecting *MAT-2* progeny on carboxin. There are only a few examples of avirulence proteins from eukaryotic pathogens that have shown a clear role in virulence. Examples include AVR-a10 and AVR-k1 from *Blumeria graminis* that enhance the penetration of the fungus in plant epidermal cells, and AVR2 and AVR4 from *C. fulvum* that inhibit an apoplastic protease and bind to fungal chitin, respectively (Dixon, et al. 1996, Joosten, et al. 1994, Ridout, et al. 2006).

Similarly, AVR3a from *Phytophthora infestans* can suppress necrotic responses in *Nicotiana benthamiana* induced by INF1 elicitor (Bos, et al. 2006).

UhAvr1 encodes an extremely monomorphic protein. This gene was sequenced from nine field isolates (six virulent and three avirulent strains) collected from different parts of the world, in addition to the parental strains. Surprisingly, only two point mutations were identified in only one avirulent strain Uh813 that translated into a single amino acid substitution. When plants R proteins recognize a particular effector from pathogens, natural selection pushes the pathogens to escape this recognition either by acquisition of additional effectors to suppress ETI, or by jettison or diversification of the recognized gene, or by disruption of gene expression. The direct interaction of an avirulence protein with a resistance protein (according to the 'receptor-ligand' model) results in diversifying selection that generates highly divergent alleles as a result of gene duplication and subsequent point mutation in order to avoid recognition by host R proteins (Dodds, et al. 2006, Ellis, et al. 2007a, Wang, et al. 2007). Twelve different alleles have been identified for *AvrL567* from six different rust strains from geographically separated locations showing 20% amino acid difference (Dodds, et al. 2006). The plant resistance locus (*L*) from flax that recognizes this gene has also undergone diversifying selection and thirteen different alleles have been identified to recognize the diverged avirulence alleles. This is consistent with an evolutionary arms race between the pathogen and the host (Dodds, et al. 2006, Ellis, et al. 1999, Stahl and Bishop 2000). Similarly, six diverged alleles of *ATR1*$^{-1NdWsB}$ have been cloned from eight strains of the oomycete pathogen, *Hayaloperonospora arabidopsidis* (Rehmany, et al. 2005). Diversifying selection also acted on *ATR13* from *H. arabidopsidis* and its corresponding locus, *RPP13*, in *Arabidopsis* (Deslandes, et al. 2003, Jia, et al. 2000).

The indirect recognition of AVR and R, the "guard model", results in purifying selection as the guard recognizes modifications of the AVR protein on the guardee and imposes selection pressure against its function (Rohmer, et al. 2004). The indirect recognition favours gene inactivation or deletion (Bent and Mackey 2007). As not many polymorphisms have been detected at the *UhAvr1* locus but have shown inactivation of the gene through the activity of transposable elements in the promoter region, I speculate that the interaction between *UhAvr1* and *Ruh1* is indirect. This needs to be confirmed experimentally, for example by using the yeast two-hybrid assay or protein pull-down after cloning of the barley *Ruh1* gene. I am not sure whether the single amino acid substitution in UhAVR1 (isoleucine to arginine) in strain Uh813 is

still recognized by the RUH1 protein or whether there is another allele of *Ruh1* in Hannchen that recognizes the mutated avirulence protein. A double-recognition strategy for at least one avirulence protein from *L. maculans*, AVRLm4-7, has been described previously in which a single amino acid substitution changes recognition by the corresponding resistance R protein (Parlange, et al. 2009).

Transposable elements can alter gene expression by insertion into a promoter element or by disrupting the protein by insertion into the ORF of the gene (Daboussi and Capy 2003, Ganko, et al. 2003, Hua-Van, et al. 2002, Kang, et al. 2001). Repetitive elements also play an important role in genome rearrangements and can result in deletion, inversion, duplication, and translocation, based on the orientation and location of the repeat on the chromosome (Daboussi and Capy 2003, Hua-Van, et al. 2000, Kim, et al. 1998, Nitta, et al. 1997, Khang, et al. 2008). The *UhAvr1* gene is located in a region of the genome that is rich in transposons and repeats and is considered an unstable part of the genome. Based on sequence analysis of the virulent and avirulent parents and the field isolates, it is likely that the activity of a TE in the promoter element of *UhAvr1* and translocation resulted in inactivation of this gene and thus the breaking of avirulence on Hannchen. Moreover, PCR and sequence analysis of BAC clones from the virulent and avirulent parents revealed a translocation of a fourteen kb-fragment including *Uhavr1* in the virulent parent to another location in the genome. Several avirulence genes from bacteria, fungi, and oomycetes have been found close to transposable elements and repeats and seem to have been inactivated by these elements (Orbach, et al. 2000, Rep, et al. 2004, Houterman, et al. 2008, Kim, et al. 1998, Rehmany, et al. 2003). Transposon-mediated mutations have been documented for several *M. oryzae* avirulence genes such as *AvrPita, AvrCo39* and *Ace1* (Bohnert, et al. 2004, Farman, et al. 2002, Farman and Leong 1998, Kang, et al. 2001, Orbach, et al. 2000, Zhou, et al. 2007). In *C. fulvum*, the insertion of a transposon resulted in truncation and inactivation of the AVR2 (Luderer, et al. 2002b). In the genome of *B. graminis* f.sp *hordei*, the *Avr-a22* and *Avr-a12* loci are close to repetitive DNA and *Avr-k1* paralogs are located near a retrotransposon (Ridout, et al. 2006, Skamnioti, et al. 2007). The insertion of a Pot3 transposon in the promoter region 302 bp upstream the ATG in *AvrPita1*, resulted in breaking its avirulence activity (Kang, et al. 2001). In *P. sojae*, an insertion of 3 kb upstream of *Avr1b-1* was suggested to be responsible for the loss of transcription in some *avr1b-1* isolates (Shan, et al. 2004).

Analysis of the 120 kb region spanning the *UhAvr1* gene in *U. hordei* revealed conserved synteny with the *U. maydis* cluster 19A. This *U. maydis* cluster is the biggest cluster of predicted secreted protein encoding genes whose transcription is induced after infection of corn inside tumor tissue; deletion of this cluster severely reduced disease on corn seedlings (Kamper 2006). The coding regions on both sides of the *UhAvr1* locus are similar to the coding regions flanking cluster 19A in *U. maydis*. It appears that both species shared some of these ancestral genes but because of their obligate biotrophic interaction with different hosts, these effectors evolved differently in the two organisms. In *U. maydis* there are several alleles of the same secreted protein coding genes and diversifying selection likely acted on these effectors to avoid host recognition, while in *U. hordei* the mechanism to avoid host recognition might have involved the activity of transposons and recombination through repetitive elements. Interestingly, in *U. maydis*-corn pathosystem, no effetor-*R* gene interaction involving avirulence and resistance genes has been genetically identified although the diversified multi-member families of effectors could indicate there might have been such an interaction in the past.

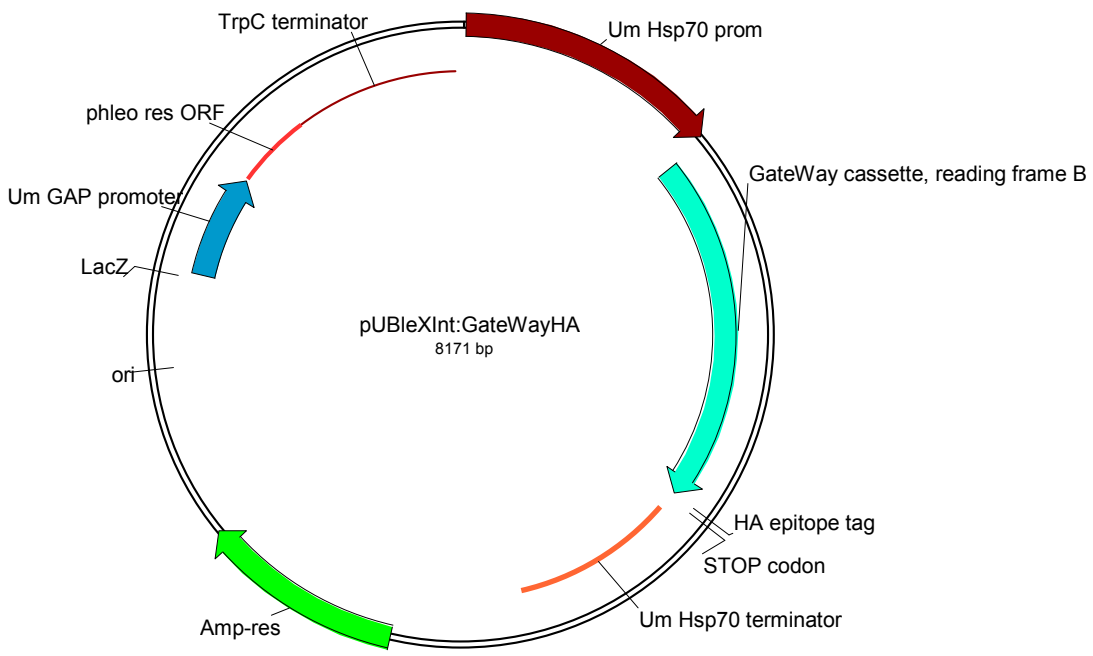

Figure 2.1 Plasmid map of pUBlexInt:GateWayHA. For the construction of pUBleX1Int:GateWayHA, a synthetic linker containing a HA epitope tag and a stop codon flanked by BamHI/BglII and KpnI cohesive ends and an Nru1 restriction enzyme site for blunt-end cloning of GateWay^{TM} recombineering cassette was used. The annealed product was ligated to a shuttle vector, cut with BamHI and Kpn1. The GateWay^{TM} cassette, reading frame B (Invitrogen) was inserted into the unique Nru1 site. Subsequently, the GateWay-HAtag-STOP cassette was amplified by PCR to clone in the shuttle vector and transferred to Ustilago-specific expression vector pUBleX1Int.

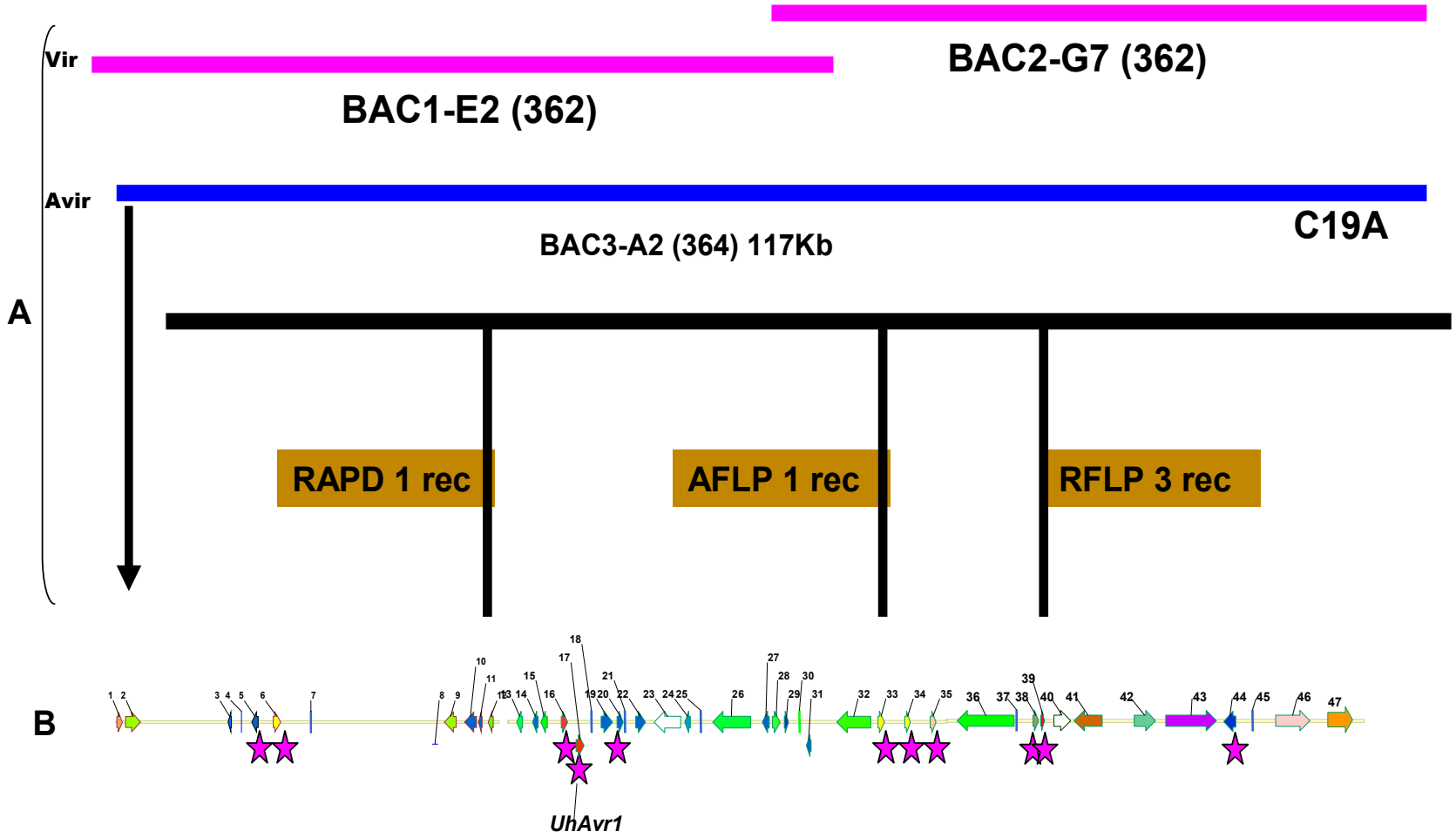

Figure 2.2 The *UhAr1* locus. (A) Three markers spanning the *UhAvr1* locus are shown by black vertical lines. BAC3-A2 from the avirulent parent (Uh364) contains the entire locus and is shown by a blue bar. The two overlapping BAC clones, 1E-2 and 2-G7 from the virulent parent Uh362, are show as pink bars at the top of BAC-3A2. (B) The entire 117 kb region from BAC3-A2 was sequenced using a transposon insertion technique. The arrows represent all the ORFs with their direction of transcription. Asterisks represent the predicted secreted proteins encoding genes at the locus.

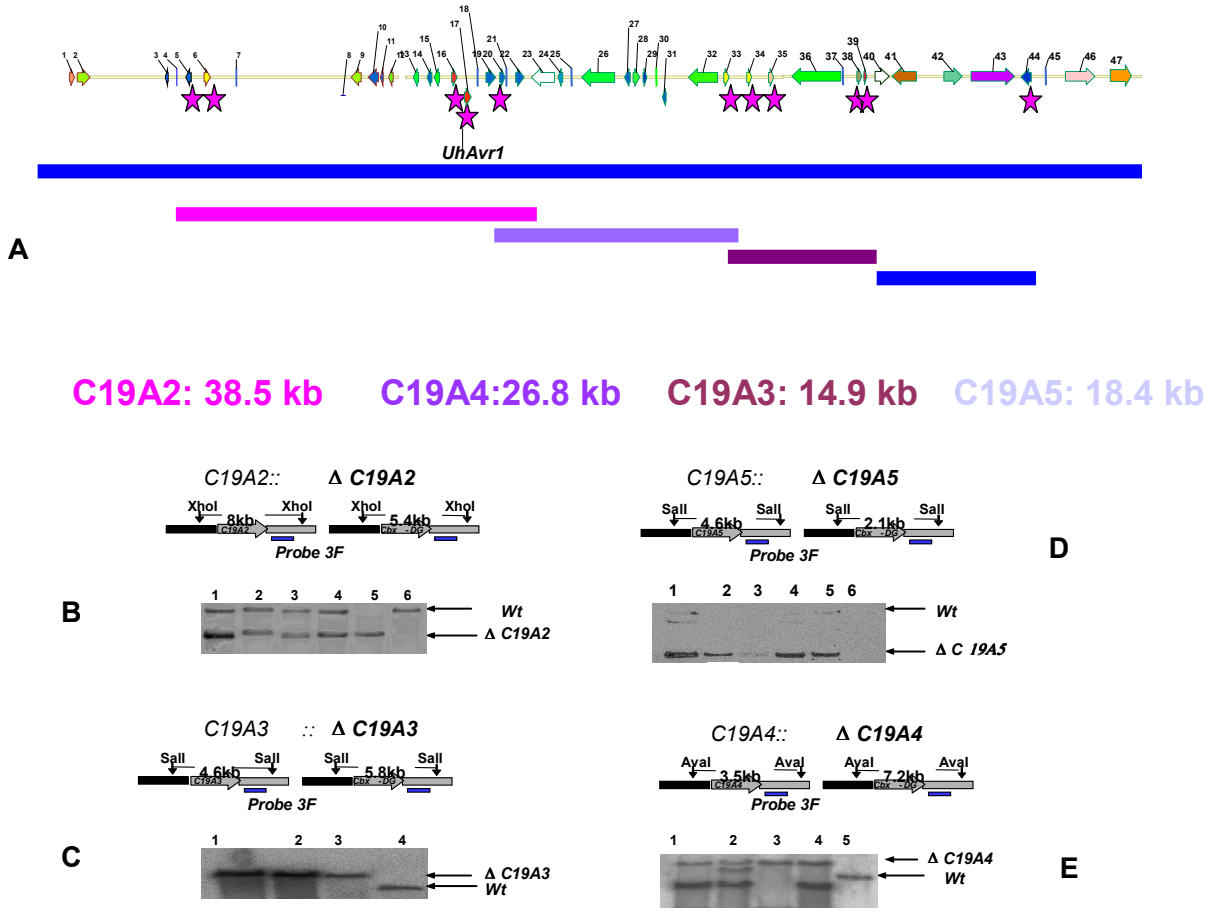

Figure 2.3 Deletion analysis of cluster C19A (A) The four overlapping bars (C19A2, C19A4, C19A3 and C19A5) under the blue bar (genomic region) represent the fragments that were deleted in the four independent deletion mutants, respectively, with their sizes in kb. (B) Southern blot analysis of C19A2 transformants: genomic DNA digested with *Xho*I. One of the transformants, number 5 in the gel shows a band of the expected size of 5.4 kb for deletion mutant and was used for pathogenicity analysis; transformants 1-4 reveal both wild-type bands and the expected fragment of deletion mutant. (C) Southern blot analysis of C19A3 transformants: genomic DNA digested with *Sal*I. All transformants reveal a band of expected size of 5.8 kb of the deletion mutant. (D) Southern blot analysis of C19A5 transformants: genomic DNA digested with *Sal*I. The analysis of these transformants revealed bands for both wild type and mutant of the expected size of 2.1 kb of deletion mutant. Two of these transformants were used for pathogenicity analysis. (E) Southern blot analysis of C19A4 transformants: genomic DNA was digested with *Ava*I. One of the transformants, lane 3, revealed a band of the expected size of 7.2 kb and was used for pathogenicity analysis; the other transformants contained one or more additional bands.

In all these Southern blot analyses, the probe used was for the 3' flank of the deletion mutant. The last sample in each gel is wild-type *U. hordei*. The little cartoon on the top of each gel is a schematic representation of the wild-type region and deletion mutant, respectively.

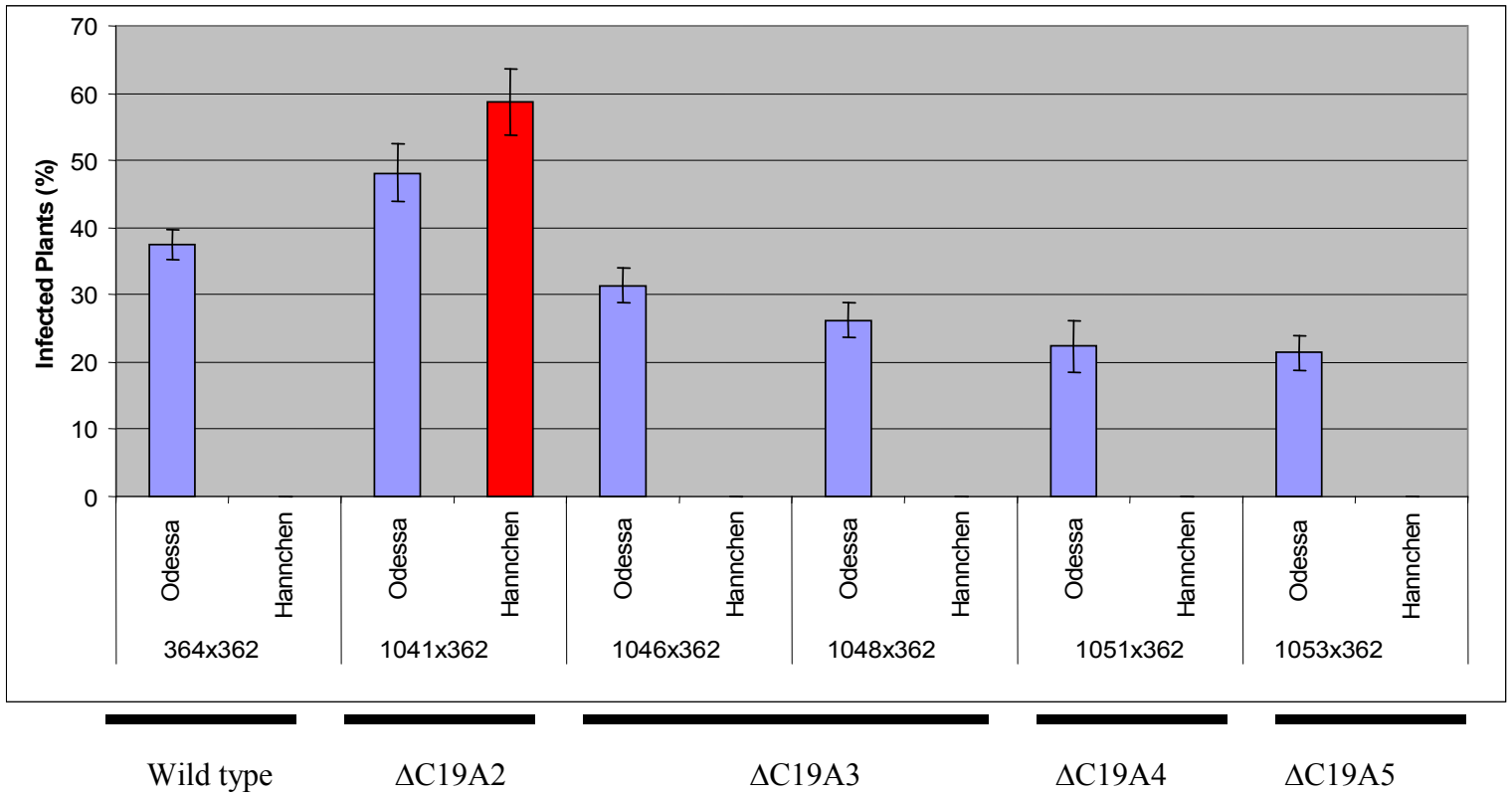

Figure 2.4 Pathogenicity test of the deletion mutants. Fragment C19A2 (deletion mutant strain Uh1041) contains the functional *UhAvr1*, as the deletion mutant of this fragment was virulent towards Hannchen, shown by the red bar. All other deletion mutants and wild type are virulent towards Odessa but avirulent towards Hannchen which shows that they have a functional *UhAvr1*. Details of all mutants and wild-type strains used in this experiment are given in Table (2.1). Bars on X-axis show the deletion mutants for different fragments mated with virulent strains and inoculated on barley cultivars Odessa and Hannchen. The Y-axis shows the percent of infected plants out of the total number of inoculated plants. The data shown here is an average of three independent experiments with standard deviation shown as error bars.

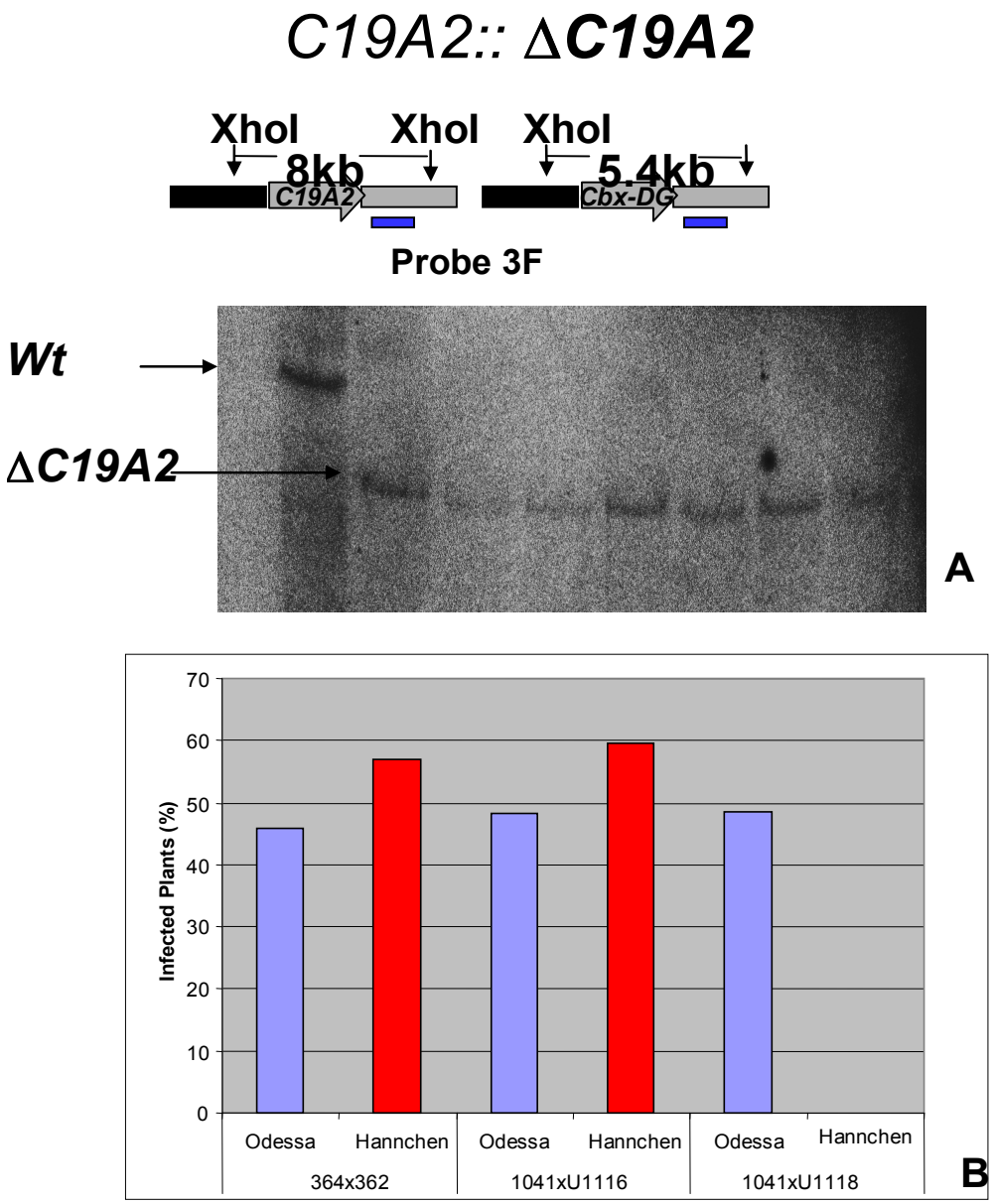

Figure 2.5 Analysis of virulence towards barley cultivars of a cross of strains both deleted for the C19A2 fragment. (A) DNA blot analysis of C19A2 deletion mutants having mating type 2. DNA was digested with *Xho*I and probed with the 3'-flank that was used for construction of the deletion construct. Two of the deletion mutants that show a band of the expected size of 5.4 kb were used for the pathogenecity tests. (B) Pathogenicity tests of crosses between mating partners both deleted for fragment C19A2. Virulence towards both barley cultivars seemed not significantly affected. Vertical bars on the X-axis show the pathogenicity of the crosses inoculated on barley cultivars Odessa (in blue) and Hannchen (in red). The Y-axis shows the percent of infected plants out of total inoculated plants.

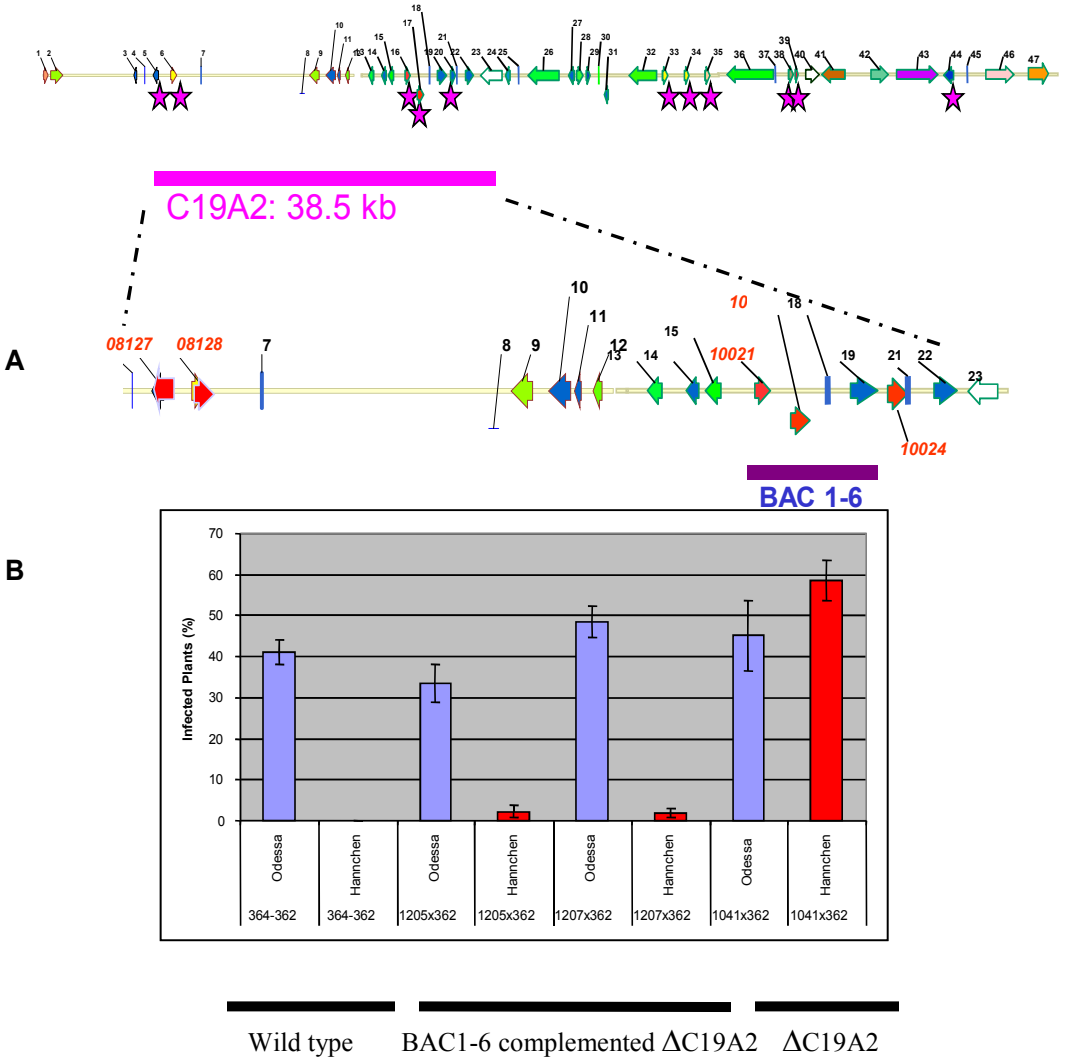

Figure 2.6 Position of BAC1-6 subclone at the *UhAvr1* locus and pathogenicity tests. (A) The 38 kb-fragment C19A2 is enlarged to show the different ORFs and predicted secreted proteins in red with arrows indicating the direction of transcription. The position of BAC1-6 is shown in by a purple bar. The figure is drawn to scale. (B) Pathogenicity test of the deletion mutant strain (Uh1041) complemented with BAC1-6 (11 kb) fragment that contains two predicted secreted proteins Uh10021 and Uh10022 as shown in A. The complemented strains cause very low disease on Hannchen while they are fully virulent towards Odessa. The deletion mutants are fully virulent towards Hannchen and the wild-type strains are virulent towards Odessa and avirulent towards Hannchen which show that they have *UhAvr1*. The details of all mutant and wild strains used in this experiment is given in the table 2.1. Bars on X-axis show different strains inoculated on barley cultivars Odessa and Hannchen. The Y-axis shows the percent of infected plants out of total inoculated plants. The data shown here is an average of three independent experiments with standard deviation as the error bar. The blue bars represent disease on Odessa and red bars represent disease on Hannchen.

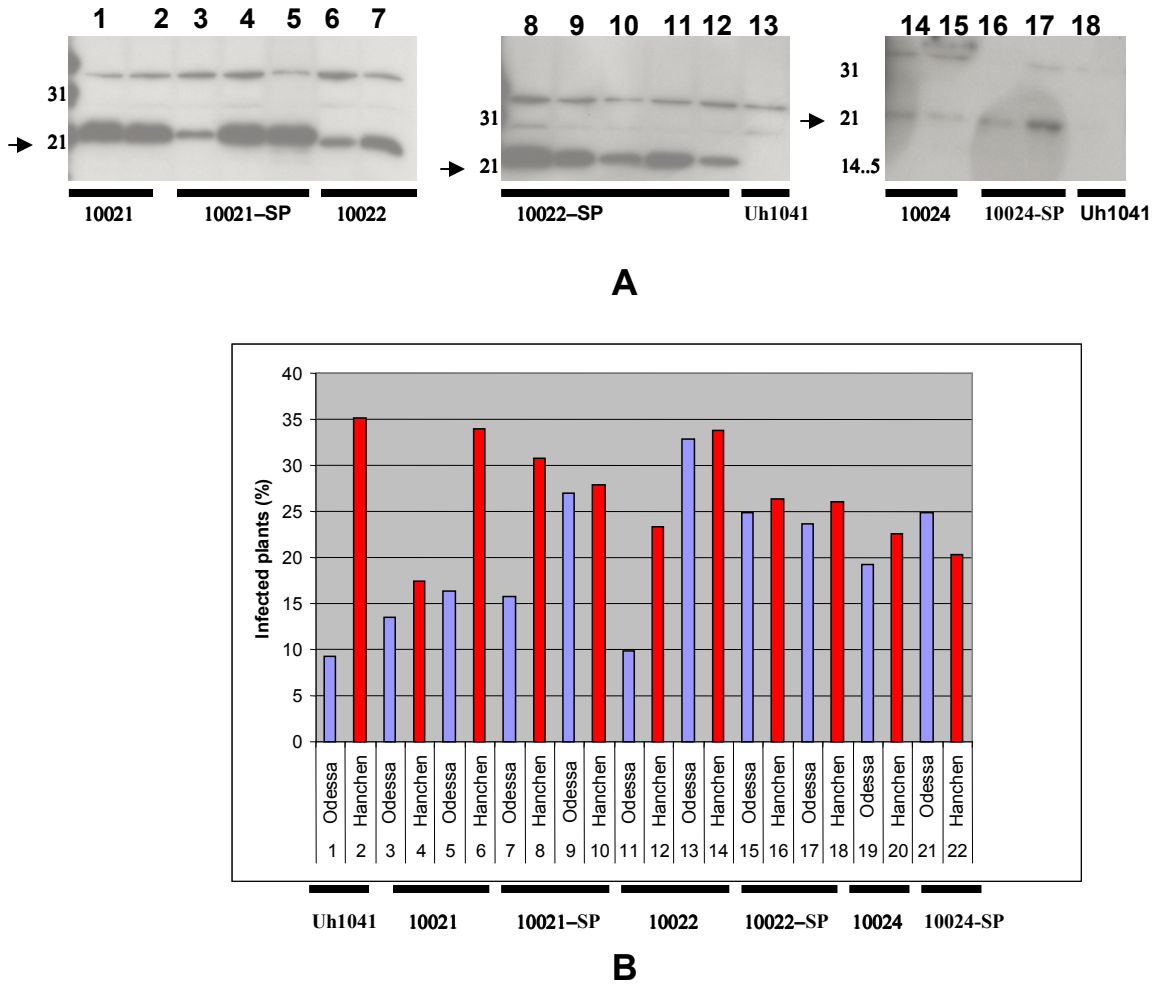

Figure 2.7 Complementation analysis of the C19A2 deletion mutant transformed with *Uh10021*, *Uh10022* and *Uh10024* and their virulence toward barley. (A) Western blot analysis of the deletion mutant Uh1041 (control; lanes 13 and 18) and Uh1041 complemented with the full length Uh10021 ORF (10021; lanes 1 and 2), Uh10022 ORF (10022; lanes 6 and 7), or Uh10024 ORF (10024; lanes 14 and 15), or with the ORFs without the signal peptide 10021-SP (lanes 3-5), 10022-SP (lanes 8-12), or 10024-SP (lanes 16 and 17) as indicated. Different lanes in the gel numbered 1-12 and 14-17, represent Uh1041 (C19A2 deletion mutant) each complemented with the respective genes shown at the bottom of the gel. On the left side of each gel the relevant protein marker is indicated in kDa. The arrows indicate the expected protein size as several non-specific bands were observed in both wild type and complemented strains. (B) Results of the pathogenicity tests with the deletion mutant strain Uh1041 and complemented with individual genes (Uh10021, Uh10022 and Uh10024) both with full length and without signal peptide under Hsp70 promoter, as indicated in the figure. The blue bars represent the percentage of infected plants of Odessa and red bars represent disease on Hannchen, given as percentage of diseased plants among the total inoculated number of plants (Y-axis).

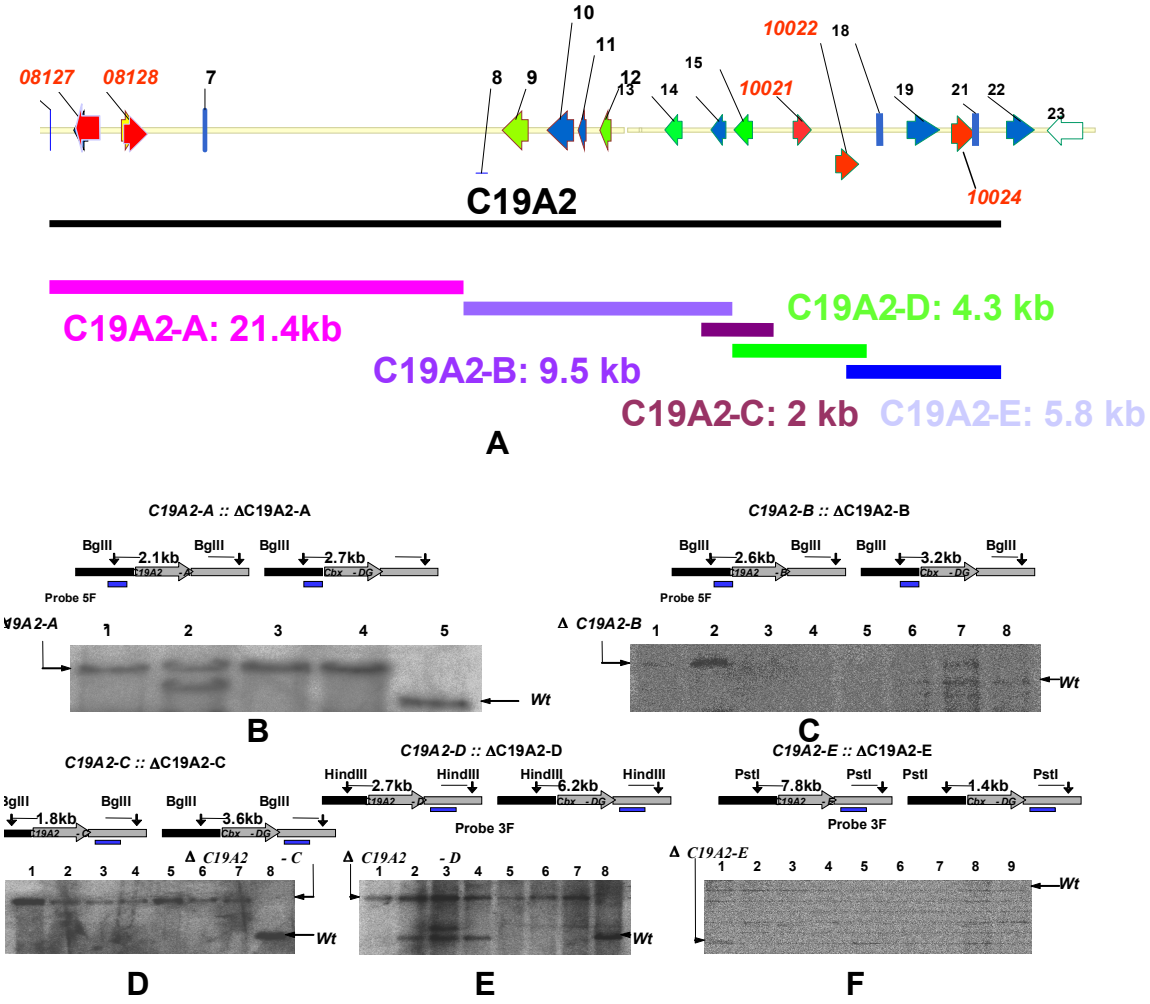

Figure 2.8 Deletion analysis of fragment C19A2. (A) Cartoon representing the fragment C19A2 (black bar) which covers the genomic region with the genes indicated (top line), and five overlapping fragments (C19A2-A, C19A2-B, C19A2-C, C19A2-D and C19A2-E, colored bars) with their sizes in kb that were deleted in five independent mutants, respectively. Wt, wild-type fragment expected (lane 6) (B) Southern blot analysis of transformants generated with deletion construct C19A2-A; total genomic DNA was digested with *Bgl*II. Three of the deletion mutants, lanes 1, 3 and 4, show a band of the expected size of 2.7 kb. One shows an additional band representing a random insertion in the genomic DNA (lane 2). Two of the mutants were used for pathogenicity analysis. (C) Southern blot analysis of transformants generated with deletion construct C19A2-B; gDNA digested with *Bgl*II. Two of the deletion mutants show a band of the expected size of 3.2 kb. (D) Southern blot analysis of transformants generated with deletion construct C19A2-C; gDNA digested with *Bgl*II. All deletion mutants analysed contain the expected size band of 3.6 kb. Two of these transformants were used for pathogenicity analysis. (E) Southern blot analysis of transformants generated with deletion construct C19A2-D; gDNA digested with *Hin*dIII. Several mutants analysed show the band of expected size of 6.2 kb while

some additionally contain the wild-type band. (F) Southern blot analysis of transformants generated with deletion construct C19A2-E; gDNA digested with *Pst*II. Several mutants analysed show the band of expected size of 6.2 kb while some additionally contain the wild-type band.

The little cartoon on the top of each gel represents a schematic representation of the wild-type region and deletion mutant, respectively. In the Southern blot analysis of the transformants generated with constructs C91A2-A and C19A2-B, the probe used was a fragment of the 5'-flank of the deletion constructs, while for C19A2-C, C19A2-D and C19A2-E, probes were taken from the 3'-flanks (indicated by little black bars under the cartoons.

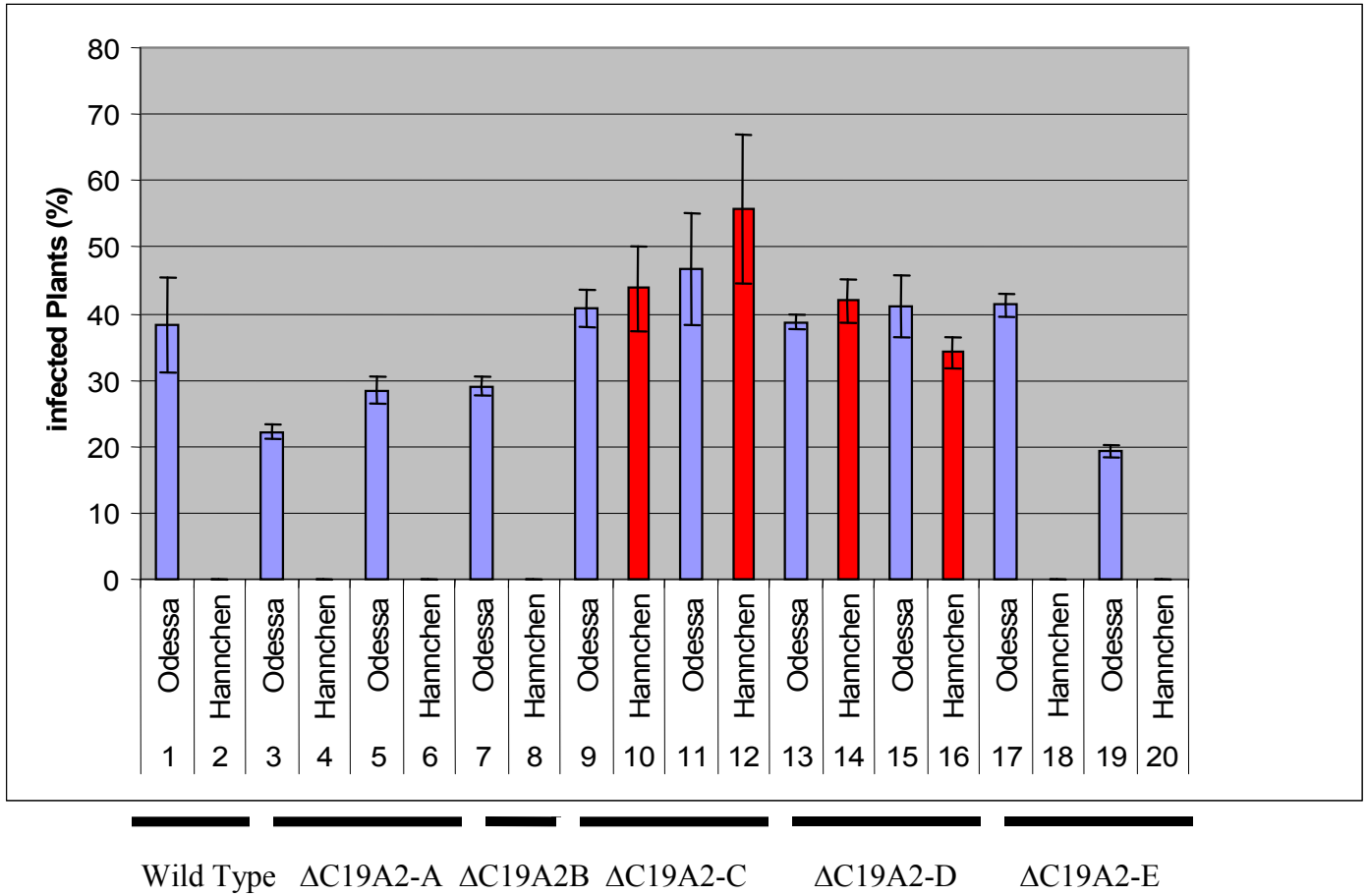

Figure 2.9 Pathogenicity test of the mutants deleted for sub-fragments of C19A2. All mutants were crossed with virulent parent Uh362. Analysis shows that the mutants deleted for fragments C19A2-C and C19A2-D were virulent towards Hannchen, shown by red bars in the figure, indicating that the functional *UhAvr1* gene is located on these fragments. All other deletion mutants and the wild type were virulent towards Odessa and avirulent towards Hannchen which showed that they had an intact *UhAvr1* gene. Details of all mutants and wild strains used in this experiment are given in the Table 2.1. Horizontal bars on the X-axis show the mutants deleted for the different fragments. The Y-axis shows the percent of infected plants out of the total inoculated plants. The data shown here is an average of three independent experiments with standard deviation as the error bar.

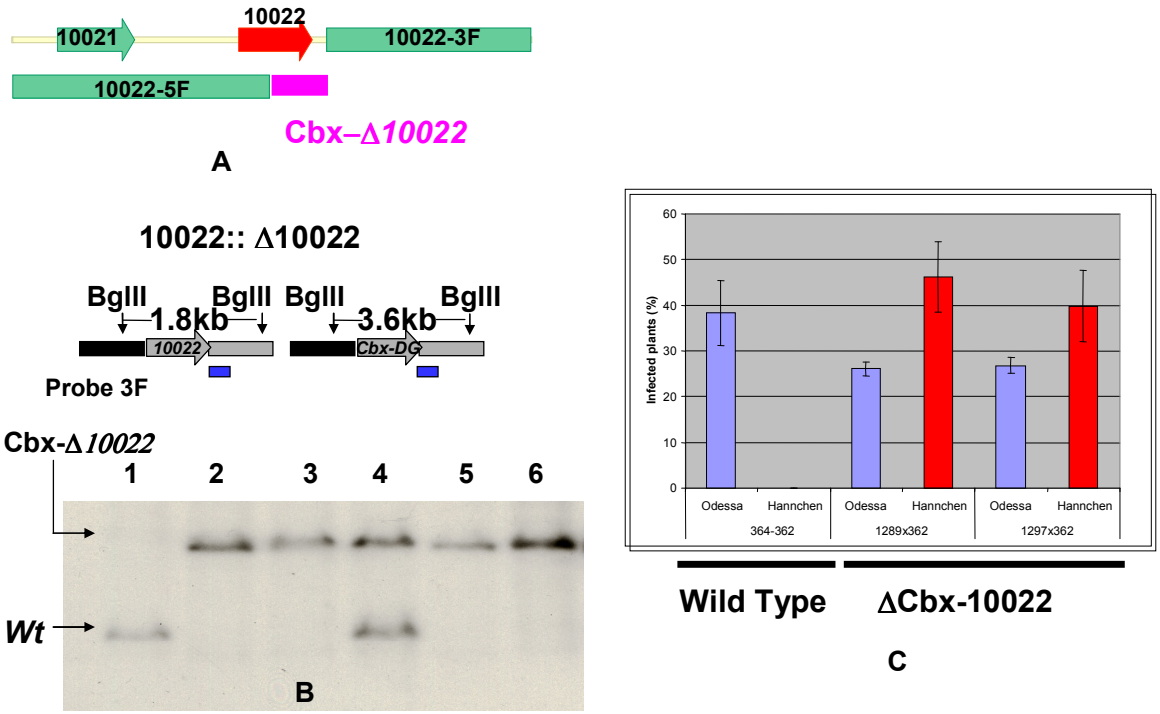

Figure 2.10 Deletion analysis of Uh10022 and pathogenicity test. (A) Schematic representation of the deletion mutant; the pink bar represents the 3'-part of Uh10022 that is deleted in deletion mutant. The green bars represent the flanks used in the deletion construct. (B) Southern blot analysis of several transformants; total gDNA was digested with BglII. Two of the deletion mutant strains showing a band of expected size of 3.6 kb, were used in pathogenicity tests. (C) All strains were mated with virulent strain Uh362 and inoculated on barley cultivars Odessa and Hannchen as indicated. Pathogenicity tests of the deletion mutants confirmed that Uh10022 is the functional *UhAvr1* gene: the mutants deleted for the 3'-end of the gene were virulent towards Hannchen, as shown by red bars in the figure. Horizontal black bars on X-axis indicate the deletion mutant and wild type. The Y-axis shows the percent of infected plants out of the total inoculated plants. The data shown here is an average of three independent experiments with standard deviation as the error bar.

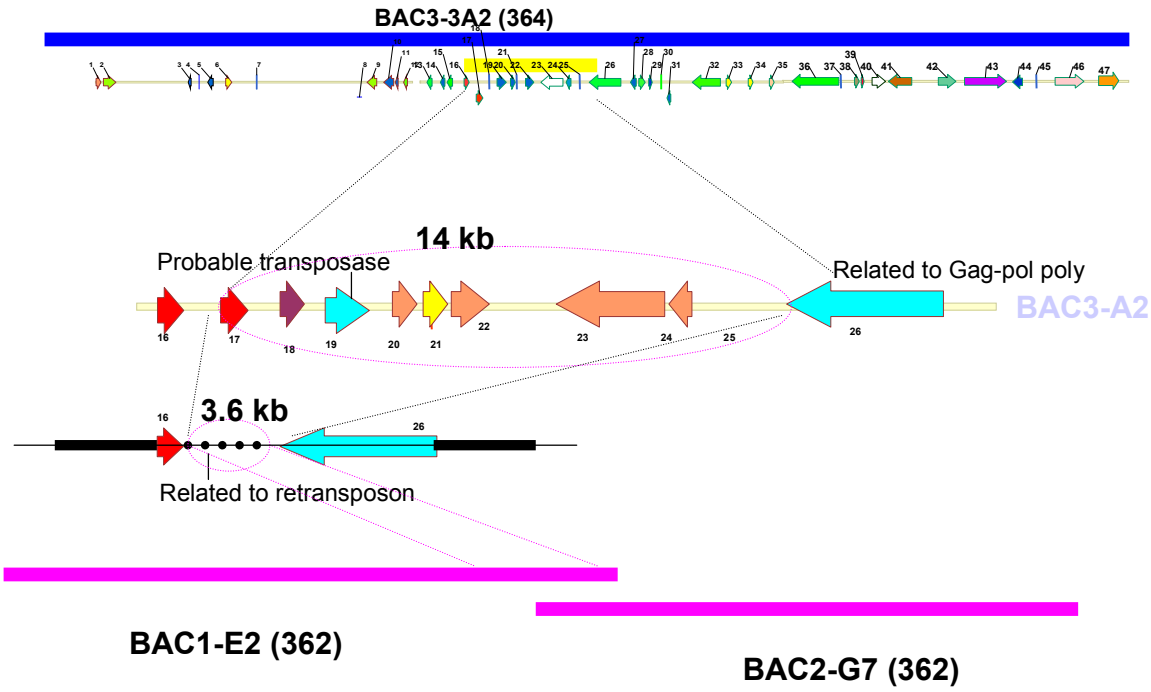

Figure 2.11 Comparison of the *UhAvr1* locus between the avirulent and virulent parents. The blue bar represents the BAC clone 3-A2 from the avirulent parent Uh364 with the genes identified underneath. The 14 kb-region containing *Uh10022* (*UhAvr1*) and 8 other ORFs, indicated by a yellow line, is enlarged below. The pink lines represent the two overlapping BAC clones from the virulent parent (Uh362). The 14 kb-region in the virulent parent is replaced by an insertion of 3.6 kb, part of which matches transposable element (TE)-related sequences shown by the dotted line in the figure. The retrotransposon was found 134 bp upstream of the *Uh10022* ATG start site.

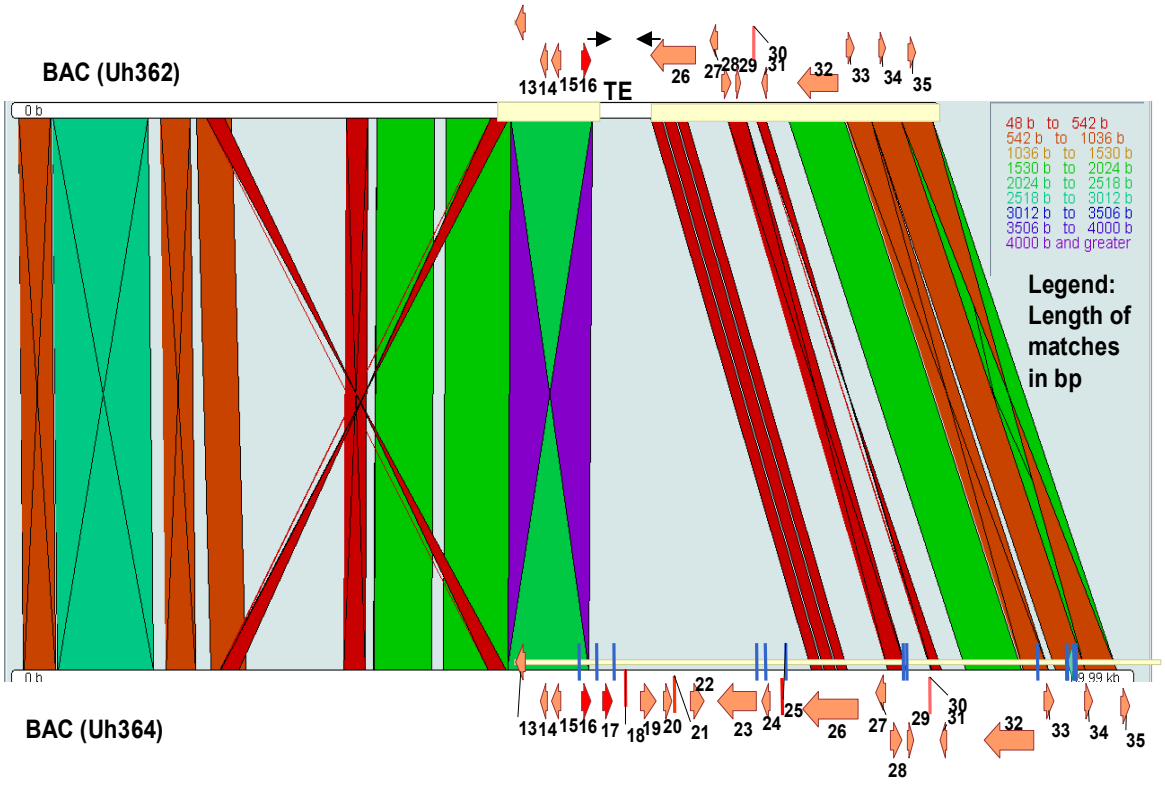

Figure 2.12 Sequence comparison of the *U. hordei UhAvr1* locus between the virulent parent Uh362 (upper) and the avirulent parent Uh364 (lower). Both loci are drawn to scale and PatternHunter output (Ma et al., 2002) was used to provide an overview of the regions being compared between the two strains. In the virulent strain, the *Avr1* gene *Uh10022* (number 17 in red in the lower panel) and seven downstream genes are absent as a deletion of a 14 kb-stretch which is present at another location in the Uh362 genome. In the virulent parent we find at this location a 3.6 kb stretch with matches to transposable element sequences as shows at the top panel (TE). Two black arrows at the upper line indicate the position of primers 1513 and 1741 in the forward and reverse orientation respectively, used for PCR to investigate the size of the insert at the *Uhavr1* locus in the virulent parent.

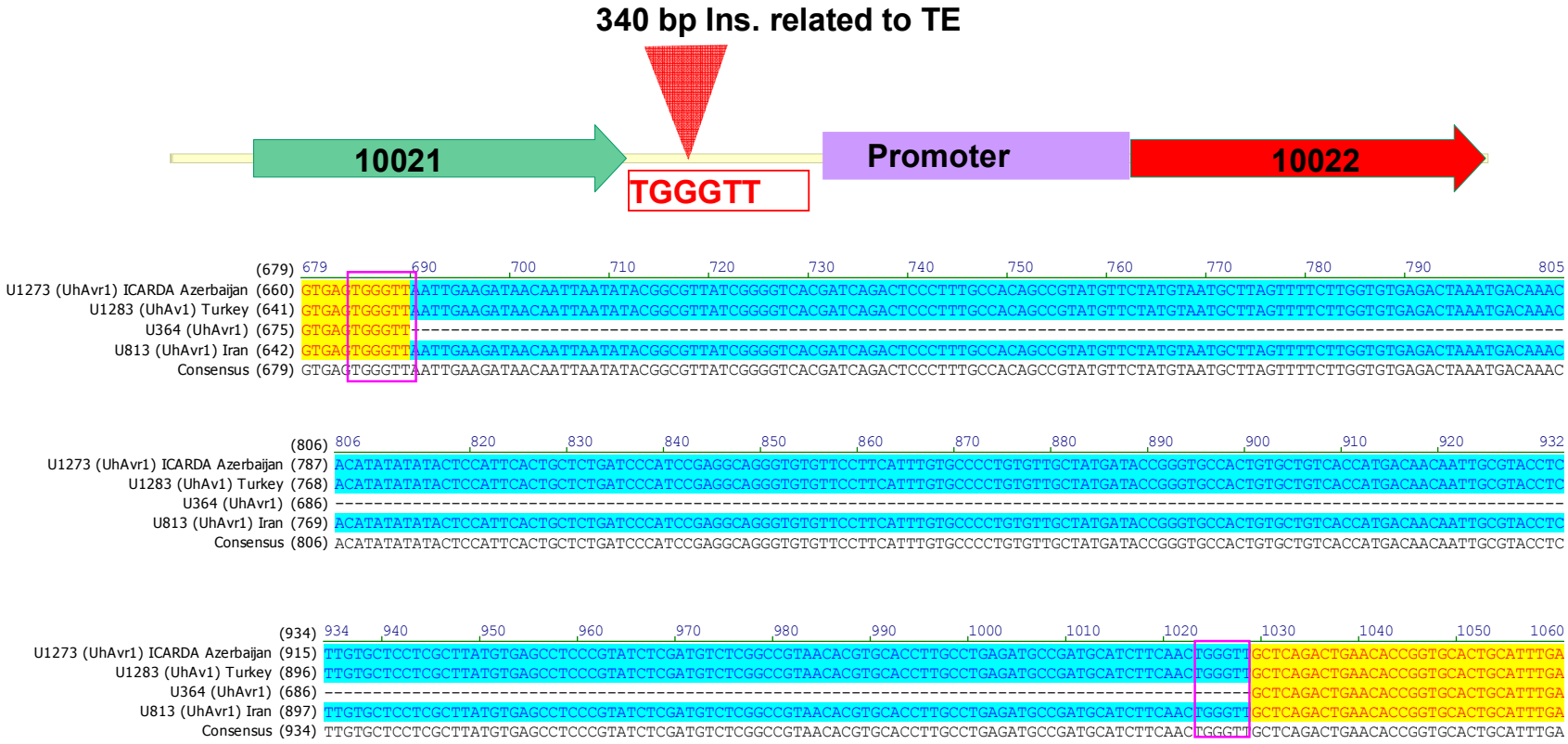

Figure 2.13 Sequence comparison of the intergenic region between *Uh10021* and *Uh10022* at the *UhAvr1* locus. Several different avirulent field isolates were compared to the avirulent parent Uh364 (see Table 2.1). Note the insertion of 340 bp, matching transposable element sequences, upstream of the *Uh10022* promoter in three avirulent field isolates (highlighted in blue). The insert is flanked by 6 bp direct repeats (TGGGTT, boxed) and this sequence matches sequences in the avirulent parental strain, possibly representing a "footprint" indicating (past) TE activity. The sequences were aligned using AlignX (Vector NTI, Invitrogen).

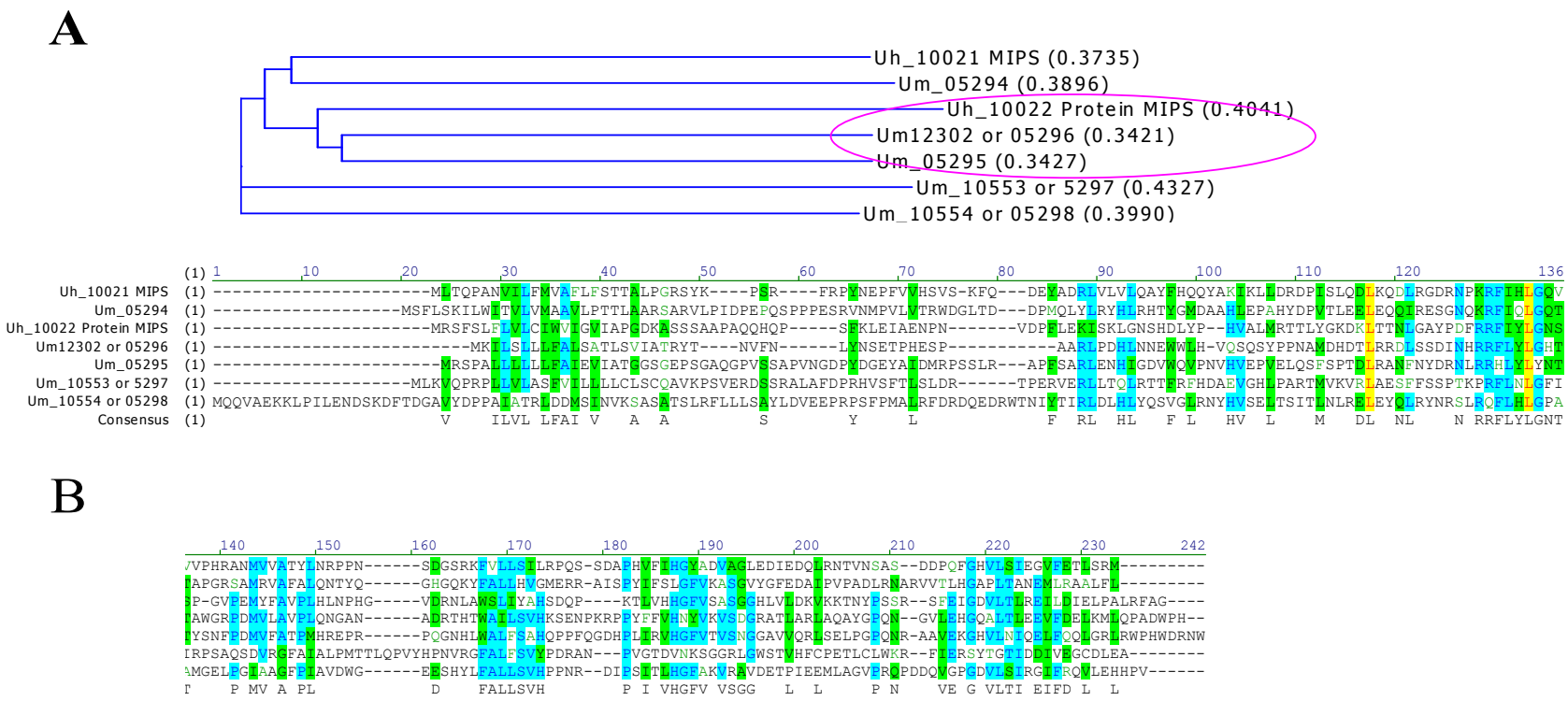

Figure 2.14 Comparison of UhAVR1 to a *U. hordei* paralog and *U. maydis* homologs. (A) A phylogeny was based on alignment of *U. hordei* UhAVR1 paralog, Uh10021, and *U. maydis* homologs, Um05294, Um05295, Um05296, Um05297 and Um05298 using the alignment from B. (B) Alignment of the protein sequences using AlignX (VectorNTI, Invitrogen).

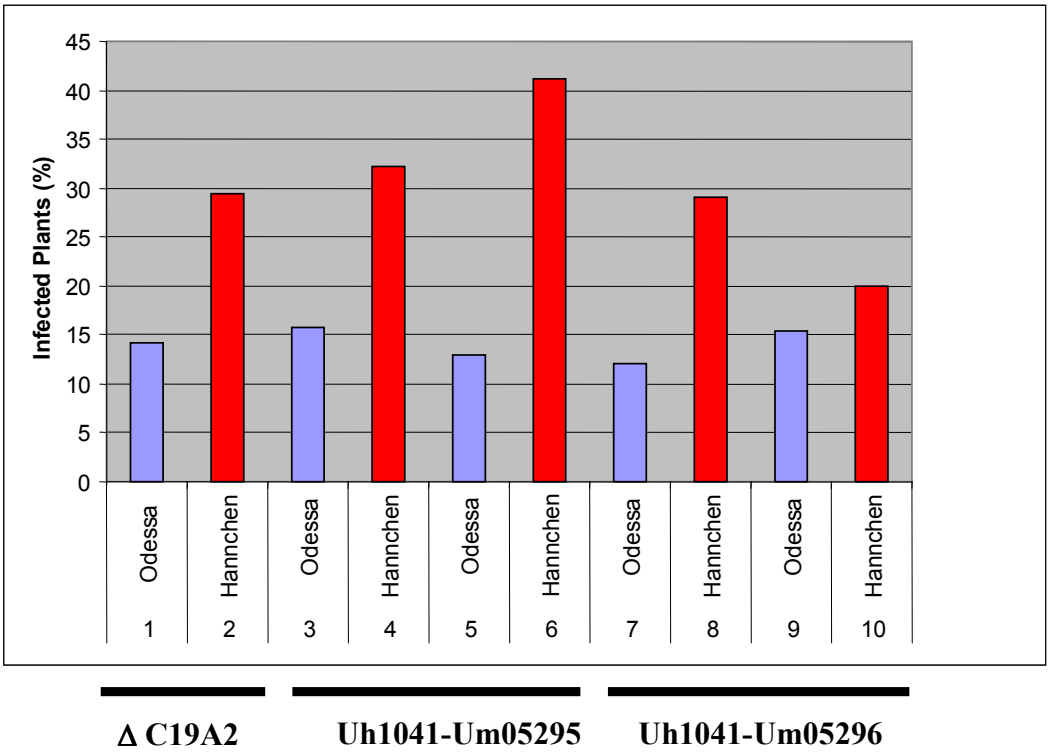

Figure 2.15 Pathogenicity tests of the C19A2 deletion mutant complemented with *U. maydis* homologs. Complemented strains and the original C19A2 deletion strain (horizontal black bars), after crossing to the virulent parent Uh362, were equally virulent towards Hannchen. Crosses were inoculated on barley cultivars Odessa (blue bars) and Hannchen (red bars). The Y-axis shows the percent of infected plants out of total inoculated plants.

Note: As complementation of this deletion mutant with *Uh10022* expressed in the same construct was not successful in restoring avirulence towards Hannchen, it is uncertain whether these *U. maydis* genes, although expressed in *U. hordei*, were properly directing protein transfer and therefore active in the host. Therefore the result of this experiment is not conclusive at this level.

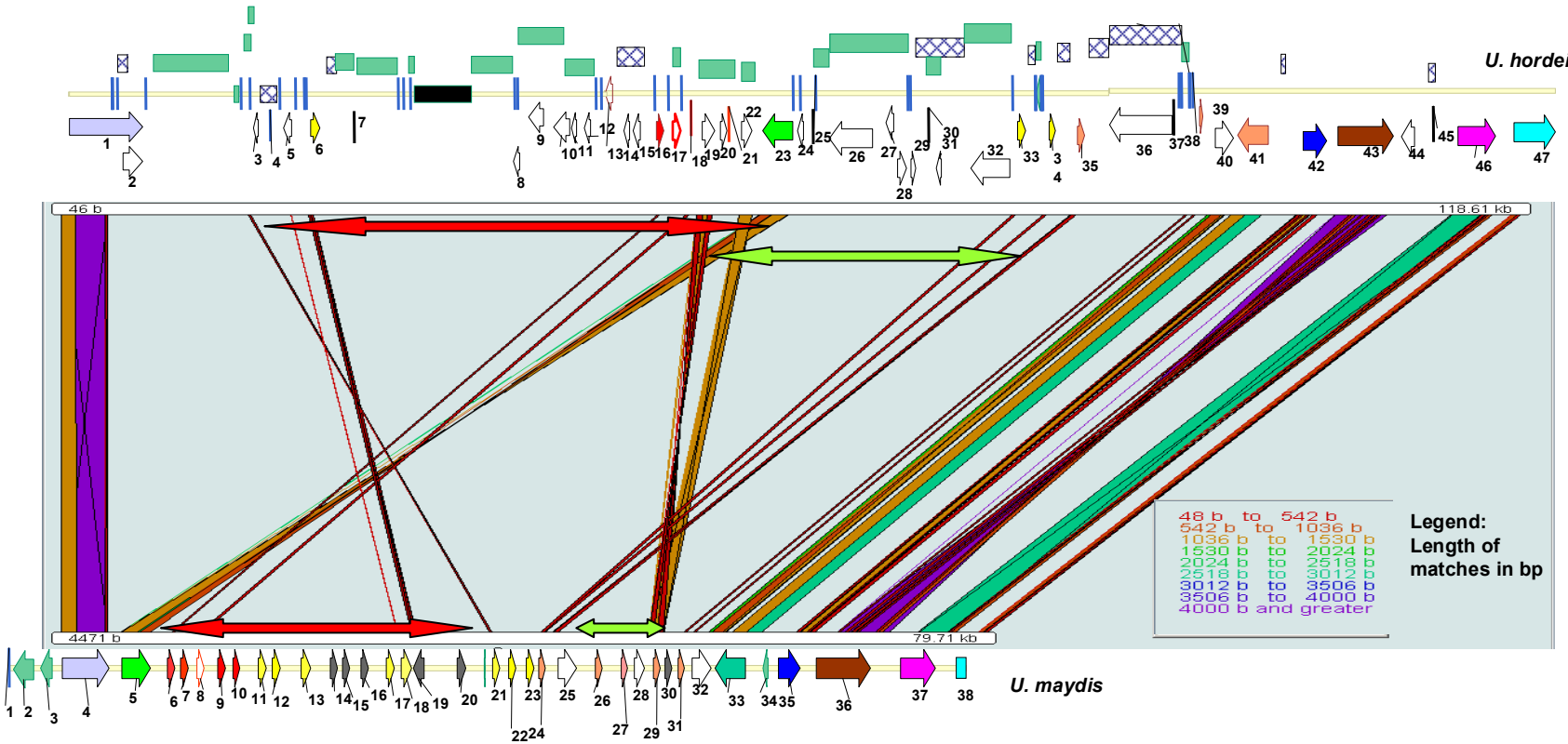

Figure 2.16 Comparison of the *U. hordei UhAvr1* locus to the syntenic region in *U. maydis* harbouring cluster 19A. The genomic *U. hordei* region, based on the sequenced BAC clone 3-A2, is at the top with arrows indicating the position and direction of transcription of the genes. Green rectangles and hatched boxes represent regions with LTRs and repeats, respectively. The red and green color two-sided-arrows represent the two fragments that are inverted in *U. hordei*. Blue vertical lines represent small repeats. The *U. maydis* sequence (bottom) was obtained from MIPS and vector NTI (Invitrogen) was used to annotate the sequences. Homologous *U. hordei* and *U. maydis* coding sequences are represented by similar colours. Gene numbering in *U. hordei* is as in Table 3.1; gene number 17 is Uh10022/*Avr1* in the top panel. Both loci are drawn to scale and PatternHunter output (Ma et al., 2002) was used to provide an overview of the regions being compared between the two species and to illustrate the gross rearrangements present in the two genomes.

Table 2.1 Strains used in this work

Strain ID	Relevant Genotype	Source or Origin
Uh364	*MAT-1 Avr1,Avr2,Avr6*	wild type; (Lining et al. 2004)
Uh365	*MAT-2 Avr1,Avr2,Avr6*	wild type; (Lining et al. 2004)
Uh359	*MAT-1 avr1,avr2,avr6*	wild type; (Lining et al. 2004)
Uh362	*MAT-2 avr1,avr2,avr*	wild type; (Lining et al. 2004)
Um324	*a2b2*	wild type; Um521 in (Kronstad and Leong 1989)
Uh1041	Uh364-Δ*19A2* (167) cbx^R	S. Ali, PhD thesis, 2011
Uh1116	Uh364- Δ*19A2* (31) cbx^R	S. Ali, PhD thesis, 2011
Uh1117	Uh364-Δ*19A2* (33) cbx^R	S. Ali, PhD thesis, 2011
Uh1118	Uh364-Δ*19A2* (35) cbx^R	S. Ali, PhD thesis, 2011
Uh1046	Uh 364-Δ*19A3* (10) cbx^R	S. Ali, PhD thesis, 2011
Uh1051	Uh364-Δ*19A4* (105) cbx^R	S. Ali, PhD thesis, 2011
Uh1053	Uh364-Δ*19A5* (2) cbx^R	S. Ali, PhD thesis, 2011
Uh1166	Uh364-Δ*19A2-A* (76) cbx^R	S. Ali, PhD thesis, 2011
Uh1173	Uh364-Δ*19A2-A* (316) cbx^R	S. Ali, PhD thesis, 2011
Uh1131	Uh364-Δ*19A2-B* (52) cbx^R	S. Ali, PhD thesis, 2011
Uh1137	Uh364-Δ*19A2-C* (19) cbx^R	S. Ali, PhD thesis, 2011
Uh1142	Uh364-Δ*19A2-C* (59) cbx^R	S. Ali, PhD thesis, 2011
Uh1149	Uh364-Δ*19A2-D* (1) cbx^R	S. Ali, PhD thesis, 2011
Uh1155	Uh364-Δ*19A2-D* (82) cbx^R	S. Ali, PhD thesis, 2011
Uh1189	Uh364-Δ*19A2-E* (64) cbx^R	S. Ali, PhD thesis, 2011
Uh1197	Uh364-Δ*19A2-E* (109) cbx^R	S. Ali, PhD thesis, 2011

Strain ID	Relevant Genotype	Source or Origin
Uh1289	Uh364-Δ*10022* (37) cbx^R	S. Ali, PhD thesis, 2011
Uh1297	Uh364-Δ*10022* (106) cbx^R	S. Ali, PhD thesis, 2011
Uh1205	Uh1041 plus BAC 1-6^{int} (2) hyg^R	S. Ali, PhD thesis, 2011
Uh1207	Uh1041 plus BAC 1-6^{int} (10) hyg^R	S. Ali, PhD thesis, 2011
Uh1212	Uh1041 plus Uh10024^{int} (6) Zeo^R	S. Ali, PhD thesis, 2011
Uh1213	Uh1041 plus Uh10024^{int} (7) Zeo^R	S. Ali, PhD thesis, 2011
Uh1268	Uh1041 plus Uh10024-SP^{int} (1) Zeo^R	S. Ali, PhD thesis, 2011
Uh1269	Uh1041 plus Uh10024-SP^{int} (2) Zeo^R	S. Ali, PhD thesis, 2011
Uh1250	Uh1041 plus Uh10021^{int} (3) Zeo^R	S. Ali, PhD thesis, 2011
Uh1251	Uh1041 plus Uh10021^{int} (4) Zeo^R	S. Ali, PhD thesis, 2011
Uh1253	Uh1041 plus Uh10021-SP^{int} (2) Zeo^R	S. Ali, PhD thesis, 2011
Uh1254	Uh1041 plus Uh10021-SP^{int} (3) Zeo^R	S. Ali, PhD thesis, 2011
Uh1255	Uh1041 plus Uh10022^{int} (1) Zeo^R	S. Ali, PhD thesis, 2011
Uh1256	Uh1041 plus Uh10022^{int} (4) Zeo^R	S. Ali, PhD thesis, 2011
Uh1257	Uh1041 plus Uh10022-SP^{int} (4) Zeo^R	S. Ali, PhD thesis, 2011
Uh1258	Uh1041 plus Uh10022-SP^{int} (6) Zeo^R	S. Ali, PhD thesis, 2011
Uh1298	Uh1041 plus Um25295^{int} (2) Zeo^R	S. Ali, PhD thesis, 2011
Uh1299	Uh1041 plus Um25295^{int} (4) Zeo^R	S. Ali, PhD thesis, 2011
Uh1301	Uh1041 plus Um25296^{int} (14) Zeo^R	S. Ali, PhD thesis, 2011
Uh1302	Uh1041 plus Um25296^{int} (4) Zeo^R	S. Ali, PhD thesis, 2011
Uh1311	Uh1041 plus Um10022 ^{int} (19) Hyg^R	S. Ali, PhD thesis, 2011

Strain ID	Relevant Genotype	Source or Origin
Uh1319	Uh1041 plus Um10022 ^{int} Hyg^R	S. Ali, PhD thesis, 2011
Uh1320	Uh1041 plus Um10022 ^{int} Hyg^R	S. Ali, PhD thesis, 2011
Uh805	*MAT-1 avr1*	Kenya
Uh815	*MAT-2 avr1*	Canary Island
Uh820	*MAT-2 avr1*	Tunisia
Uh795	*MAT-1 Avr1*	unknown
Uh813	*MAT-1 Avr1*	Iran
Uh822	*MAT-1 avr1*	Canada
Uh811	*MAT-1 avr1*	Ethiopia
Uh818	*MAT-1 avr1*	Spain
Uh1273	*MAT* (unknown) *Avr1*	ICARDA Azerbaijan
Uh1283	*MAT* (unknown) *Avr1*	Turkey

Strain ID refers to Bakkeren Lab inventory, Uh for *U. hordei* and Um for *U. maydis.* All mutants were generated in the Uh364 background as indicated. R, resistant to the indicated antibiotic: Hyg, hygromycin B; Zeo, zeocin; cbx, carboxin. ^{int}, integrative complementing plasmid. The number in parentheses represents the specific transformant chosen for analysis. Δ:deletion mutant, indicating specific gene or region.

Table 2.2 Primers used in this work

#	Name of the primer	Sequence	Purpose
1244	UH_13897_Fw	**CACC**ATGCTTACTCAACCGGCCAAC	Cloning of Uh10021
728	Uh05294plus_f	GGAACTGTGCTCTGGTAGTGG	Sequencing of Uh10021
1246	UH_13897_Rev	CATTCGTGACAACGTCTCAAAAAC	Cloning and sequencing of Uh10021
1245	UH_13897-SP_FW	**CACC**ATGGCACTACCCGGTCGCAGCTAC	Cloning of 10021
1247	UH_10022_Fw	**CACC**ATGCGATCGTTTTCCCTTTTCC	Cloning and sequencing of Uh10022
1248	UH_10022-SP_Fw	**CACC**ATGCCTGGCGACAAAGCTTCTTC	Cloning of Uh10022
1249	UH_10022_Rev	TCCGGCAAATCGGAGCGCAG	Cloning and sequencing of Uh10022
736	Uh05311aPlus_f	GCTTTTCATCAGAGCCATACCT	Sequencing of Uh08130
737	Uh05311aPlus_r	TGGCTTGTTTACAGAGTGCAA	Sequencing of Uh08130
1253	UH_08132_Fw	CACCATGGCCACAACATCACTCTTAC	Sequencing of Uh08132
1281	Uh08132-plus-L	CGCTATGGAAGCACTTCATTTT	Sequencing of Uh08132
661	Uh5311R2	GCATTCGGCCTGATACCAAC	Sequencing of Uh08132
1256	UH_08128_Fw	CACCATGCTTTTCTTTATTCTCGCC	Sequencing of Uh08128
1258	UH_08128_Rev	AGAAAAGTGCGGCAGTGATGC	Sequencing of Uh08128

#	Name of the primer	Sequence	Purpose
730	Uh05306plus_f	ATAGCCCTCCTTCATGGTGT	Sequencing of Uh08128
731	Uh05306plus_r	TCGCAGTGCCTTTCATATTG	Sequencing of Uh08128
1272	Uh08127-plusL	GCAGAGCATGACGTGAAACTAC	Sequencing of Uh08127
1273	Uh08127-plusR	GTTCAAGGCCCTATATCCCTCT	Sequencing of Uh08127
1261	UH_08127_Rev	TCCGTGGCCTCTAACAGCAAG	Sequencing of Uh08127
1265	UH_08139_Fw	CACCATGTTCCGAATCGGCTTTGTC	Sequencing of Uh08139
1267	UH_08139_Rev	TCCAGGCAATCTGATCAGGC	Sequencing of Uh08139
727	Uh05318homo_r	GCGCACTAGTTCAATTCAAAGCTGGAGGTATG	Sequencing of Uh10033
966	5319 plus_r	TCTCCCTTCCTCGTCAACCTG	Sequencing of Uh10033
1282	Uh10033-plus-L	GGTCAAGTCGACCTCCAACAG	Sequencing of Uh13916
1283	Uh10033-plus-R	GTCCCTTCCGTCACTTCCAT	Sequencing of Uh13916
1277	Uh10024-plusL	TGAGATCTGTCATAGAGCTGTTTC	Sequencing of Uh10024
1278	Uh10024-plusR	GCATCTTCGGATGTCAGGTAGT	Sequencing of Uh10024
1424	UH_10024_Fw	**CACC**ATGTTGCCTGCAACACTGCCTTC	Cloning of Uh10024
1425	UH_10024_rev	CAGCATCGCCAGCGATGCTGCTC	Cloning of Uh10024

#	Name of the primer	Sequence	Purpose
1510	UH_10024-SP_Fw	**CACC**ATGCCTGGATGCTCAAGAAGCAGGAG	Cloning of Uh10024
1152	UH_08134_Fw	CACCATGAAGGTACATCTGTCTAC	Sequencing of Uh08134
1154	UH_08134_Rev	GCCTCGAATGGTCACAGG	Sequencing of Uh08134
1284	C19-A1-5L-1Sce-1F	**AAAATAGGGATAACAGGGTAAT**CGACAGATCTCGAGGAAACC	5F of Deletion construct C19A2
1285	C19-A1-5R-flank–attB1	**GGGGACAAGTTTGTACAAAAAAGCAGGCTAT**TGAATTGTTTGCCACACCTG	5F Deletion construct C19A2
1286	C19-A1-ko-5 FlankL	TCACTTCAGGAGGTGATCAAGA	Confirmation of deletion mutant C19A2
1289	C19-A2-3L-attB2	**GGGGACCACTTTGTACAAGAAAGCTGGGTA**GGAGAGAAGAAGCAGAGCT	3F of Deletion construct C19A2
1290	C19-A2-3R-1Sce-1R	**AAAAATTACCCTGTTATCCCTA**TTGTGCTTCACTGCACCTTC	3F of Deletion construct C19A2
1291	C19-A2-ko-3 FlankR	TCCCTGTCGGTGTCTTCTTACT	Confirmation of deletion mutant C19A2
1292	C19-A3-5L-1sce-1F	**AAAATAGGGATAACAGGGTAAT**CCTGTCGATTGCTAGGAAGG	5F Deletion construct C19A3
1293	C19-A3-5R–attB1	**GGGGACAAGTTTGTACAAAAAAGCAGGCTAT**TGAGGGTCAATCGGAGAGAT	5F Deletion construct C19A3
1294	C19-A3-ko-5 FlankL	TTGTTGTCTTGGTTTCCTGTGT	Confirmation of deletion mutant C19A2-A
1295	C19-A4-5L-1Sce-1F	**AAAATAGGGATAACAGGGTAAT**AAGCCCTGCTTCTTCTCTCC	3F of Deletion construct C19A3
1296	C19-A4-5R-attB1	**GGGGACAAGTTTGTACAAAAAAGCAGGCTAT**GAGTGGATCCCCATTGTCAT	3F of Deletion construct C19A3
1297	C19-A4-ko-5 FlankL	AGCTTGCAGTCTGTTCATCATC	Confirmation of deletion mutant C19A5

#	Name of the primer	Sequence	Purpose
1298	C19-A4-3L attB2	**GGGGACCACTTTGTACAAGAAAGCTGGGTA**CGTACAGGACCGTGAGGACT	5F Deletion construct C19A4
1299	C19-A4-3R-1Sce-1-R	**AAAAATTACCCTGTTATCCCTA**GTGGATCAGCTGTTCACTCG	5F Deletion construct C19A4
1300	C19-A5-5L-1Sce1-F	**AAAATAGGGATAACAGGGTAAT**CCCCCTATCTGGCTCTCTTC	5F Deletion construct C19A5
1301	C19-A5-5R-attB1	**GGGGACAAGTTTGTACAAAAAAGCAGGCTAT**GAACGTGTGGTATGCTGAGG	5F Deletion construct C19A5
1302	C19-A5-3L-attB2	**GGGGACCACTTTGTACAAGAAAGCTGGGTA**GATCGTGGCTCCAAGACAGT	3F Deletion construct C19A5
1303	C19-A5-3R-1Sce-1R	**AAAAATTACCCTGTTATCCCTA**GGACTGGACTTTAGGGCACA	3F Deletion construct C19A5
1428	C19A2-A-3F-attB2-L	**GGGGACCACTTTGTACAAGAAAGCTGGGTA**GCATTGTGCTCAAGCTGTGT	3F Deletion construct C19A-A
1429	C19A2-A-3F-1sce1R-R	**AAAAATTACCCTGTTATCCCTA**ACTGCTGGGCAAGAATGACT	3F Deletion construct C19A-A
1430	C19A2-b-5F-1sce1F-L	**AAAATAGGGATAACAGGGTAAT**CTCAAACCCAATCTGCAGTG	5F Deletion construct C19A-B
1431	C19A2-b-5F-attB1-R	**GGGGACAAGTTTGTACAAAAAAGCAGGCTAT**AGGTTAGCGGTCCAGATCAA	5F Deletion construct C19A-B
1432	C19A2-b-3F-attB2-L	**GGGGACCACTTTGTACAAGAAAGCTGGGTA**CTAGGACGAAACAGCCAAGC	3F Deletion construct C19A-B
1433	C19A2-b-3F-1SceR-R	**AAAAATTACCCTGTTATCCCTA**ACTCCAATCACGGGAATCAC	3F Deletion construct C19A-B
1434	C19A2-c-5F-1sce1F-L	**AAAATAGGGATAACAGGGTAAT**TGGGTAGAGGTTTGGTGAGG	5F Deletion construct C19A-C
1435	C19A2-c-5F-attB1-R	**GGGGACAAGTTTGTACAAAAAAGCAGGCTAT**AAGAATCCTGCCTTGCTTCA	5F Deletion construct C19A-C
1436	C19A2-c-3F-attB2-L	**GGGGACCACTTTGTACAAGAAAGCTGGGTA**CCTTAGCCTAGTCCCGCTCT	3F Deletion construct C19A-C

#	Name of the primer	Sequence	Purpose
1437	C19A2-c-3F-1SceR-R	**AAAAATTACCCTGTTATCCCTA**GAGAAGAAGCAGGGCTTTCA	3F Deletion construct C19A-C
1438	C19A2-d-5F-1sce1F-L	**AAAATAGGGATAACAGGGTAAT**TTCATCTTCGCCCATTCTTC	5F Deletion construct C19A-D
1439	C19A2-d-5F-attB1-R	**GGGGACAAGTTTGTACAAAAAAGCAGGCTAT**TTGAAGCTCCTCGTCAGACA	5F Deletion construct C19A-D
1440	C19A2-d-3F-attB2-L	**GGGGACCACTTTGTACAAGAAAGCTGGGTA**CATCATCATAGGCTGAGTGGA	3F Deletion construct C19A-D
1441	C19A2-d-3F-1SceR-R	**AAAAATTACCCTGTTATCCCTA**GGCAAGCTTTGACTTGGAAT	3F Deletion construct C19A-D
1446	C19A2-e-3F-attB2-L	**GGGGACCACTTTGTACAAGAAAGCTGGGTA**GAGACGATCGTGCGTATGTG	3F Deletion construct C19A-E
1447	C19A2-e-3F-1SceR-R	**AAAAATTACCCTGTTATCCCTA**TTCACTGCGATCTGCCATAG	3F Deletion construct C19A-E
1506	C19A2-A-KO-3F	TTACAATTGCAGGCAACCAG	Confirmation of deletion mutant C19A2-A
1507	C19A2-B-KO-5F	GCATATGGCTTCTTGCCATT	Confirmation of deletion mutant C19A2-B
1508	C19A2-D-KO-3F	TGTCATACAGCCCCAGATCA	Confirmation of deletion mutant C19A2-D
1551	C19A2-E-ko-3F	TGATGCTCATGCTGATTTCA	Confirmation of deletion mutant C19A2-D
1614	Uh10022-5F-attBI	**GGGGACAAGTTTGTACAAAAAAGCAGGCTAT**AGGTTAGTGGTCAGTTTATC	5F Deletion construct Uh10022
1615	Uh10022F-for RNAi	CACCGTGCACCATGGATTCGTCT	
1616	LP-10022 with Pro-NotI	**ggtagcggccgc**AACGTTTGTTCAGCCCTGTT	Cloning of Uh10022 in pHYG101
1617	RP-10022 with Pro-NotI	**ggatgcggccgc**TCCGGCAAATCGGAGCGCAG	Cloning of Uh10022 in pHYG101

#	Name of the primer	Sequence	Purpose
1632	Um5295F	**CACC**ATGAGATCTCCCGCTCTTC	Cloning Um05295 in pUBleX1Int:GateWayH
1633	Um5295R	CCAGTTGCGATCCCAATGGG	Cloning of Um05295 in pUBleX1Int:GateWayHA
1634	Um5296F	**CACC**ATGGGCAAAGCAACAGAGAT	Cloning of Um05296 in pUBleX1Int:GateWayHA
1635	Um5296R	ATGAGGCCAGTCGGCTGGCT	Cloning of Um05296 in pUBleX1Int:GateWayHA
1513	VirC17R1	CTGCAGGTCGACTCTAGAGG	For PCR of transposable element
1741	10026-60K-REV	GGGAAGACCAACAACCGACA	For PCR of transposable element
1685	10021F1(QPCR)	CGATGTAGCGGGTCTCGAAG	For QPCR analysis
1686	10021R1(QPCR)	CCCCTTCGATCGAGAGAACA	For QPCR analysis
1687	10021F2(QPCR)	ATCTTCTGATGCCCCACACG	For QPCR analysis
1688	10021R2(QPCR)	CGAGGCAGAGTTCACGGTGT	For QPCR analysis
1689	10022 qPCR-L 1	GGTGGACACCTGGTCCTAGA	For QPCR analysis
1690	10022 qPCR-R 1	CTGAGGGTCAGAACGTCTCC	For QPCR analysis
1690	10022 qPCR-R 1	CTGAGGGTCAGAACGTCTCC	For QPCR analysis
1692	10022 qPCR-R 2	GCAGTTCAATATCAAGTATCTCTCTG	For QPCR analysis
1595	Uh-0772 eIFB2	AAATGGTGTCCGCTCATCTC	For QPCR analysis

#	Name of the primer	Sequence	Purpose
1596	Uh-0772 eIFB2	CAACCCACGATGTTCTCCTC	For QPCR analysis
1604	Uh-08813 Actin	TCGATCCTTCGTCTCGATCT	For QPCR analysis
1605	Uh-08813 Actin	CAGAGCCGAAGACTGGGTAG	For QPCR analysis
1668	10026F	TGTCGGTTGTTGGTCTTCCC	For PCR amplification
1669	10027R	TGATCAACCACATGGGTGCT	For PCR amplification
1670	10028F	CCAGTAGCCTGGAAGTCAGC	For PCR amplification
1671	10028R	TAGACTTTCGTGCGTTGTGC	For PCR amplification
1672	13901F	GAATTTCCGAGTCGATCCAA	For PCR amplification
1673	10030R	GCAAGAGGGAGCAACAAGTC	For PCR amplification

F, forward. R, reverse. 3F and 5F indicates that primers were used for the amplification of 3'- and 5'-ends of deleted regions. The I-SceI recognition sequence is in bold type and underlined, while only bold type represents the *attB1* and *attB2* sequences on the primers used for the deletion constructs. The tetranucleotide CACC in bold type indicates the sequence used for directional cloning in the pENTR/D^{TM} Gateway plasmid (Invitrogen). Lower case in bold type on primers 1616 and 1617 represents the restriction recognition sequence for NotI enzymes. #; Bakkeren Lab inventory primer number.

Table 2.3 *U. hordei* genes located on BAC 3-A2 (117 kb) and their homologs in *U. maydis*

Number [1]	*U. hordei* MIPS ID [2]	*U. maydis* homolog [3]	ExpValue [4]	Percent Identity [5]	Percent Similarity [6]	score/selfscore ratio query [7]	Function [8]
1	UH_08121	um05292	0	82.8	94.4	0.85	related to DigA protein
2	UH_13886	um10151	2.09E-38	42.9	63.9	0.15	hypothetical protein
3	UH_13887	No hit in Um					
4	UH_13888	um04317	0.04	36	52	0.13	hypothetical protein
5	UH_08127	um04656.2	0.02	26.4	45.5	0.06	hypothetical protein
6	UH_08128	um05306	1.54E-44	34.2	62.6	0.28	conserved hypothetical Ustilaginaceae-specific protein
7	UH_13890	um00543	0.01	36.4	56.8	0.14	hypothetical protein
8	UH_10014	um02565	0	62.4	88.6	0.54	conserved hypothetical protein
9	UH_10015	um12288	0	33.5	56.3	0.29	conserved hypothetical protein
10	UH_10016	um02285	4.60E-05	20.3	54.2	0.05	hypothetical protein
11	UH_13893	um11726	0.01	25	52	0.1	hypothetical protein
12	UH_10017	um02745	2.39E-16	30.6	57.5	0.23	conserved hypothetical protein
13	UH_10018	um00776	6.96E-18	46.1	68.5	0.18	conserved hypothetical Ustilaginaceae-specific protein
14	UH_10019	um06249	8.76E-05	25.3	53.2	0.11	hypothetical protein
15	UH_10020	um02747	4.95E-12	25.8	60.3	0.14	conserved hypothetical protein

Number [1]	*U. hordei* MIPS ID [2]	*U. maydis* homolog [3]	ExpValue [4]	Percent Identity [5]	Percent Similarity [6]	score/selfscore ratio query [7]	Function [8]
16	UH_10021	um05294	2.28E-15	27.8	59.3	0.17	conserved hypothetical Ustilaginaceae-specific protein
17	Uh_10022	um12302	1.87E-16	30.5	65.2	0.18	conserved hypothetical Ustilaginaceae-specific protein
18	Uh_13899	No hit in Um					
19	UH_10023	um04172.2	0.00066	18.7	56	0.05	probable transposase
20	UH_10024	um03280	0.00027	29.4	51	0.09	hypothetical protein
21	Uh_13901	No hit in Um					
22	UH_10025	um06075	0.00808	27	57	0.05	probable transposase
23	UH_08123	um05293	0	91.6	97.2	0.9	probable oligosaccharyltransferase
24	Uh_13903	No hit in Um					
25	Uh_13904	No hit in Um					
26	UH_10026	um04367	0	58.3	75.4	0.12	related to Gag-pol polyprotein
27	UH_10027	um10618	1.10E-11	32.9	71.4	0.12	hypothetical protein
28	UH_10028	um02565	0	63	87.3	0.49	conserved hypothetical protein
29	Uh_13907	No hit in Um					
30	Uh_10029	No hit in Um					
31	UH_10030	um05274	0.02	31.2	64.1	0.1	hypothetical protein

Number [1]	*U. hordei* MIPS ID [2]	*U. maydis* homolog [3]	ExpValue [4]	Percent Identity [5]	Percent Similarity [6]	score/selfscore ratio query [7]	Function [8]
32	UH_10031	um02565	0	46.1	63	0.12	related to retrotransposon protein
33	UH_08130	um05311	5.89E-21	36.8	67.2	0.21	conserved hypothetical Ustilaginaceae-specific protein
34	UH_08132	um05311	1.97E-21	33.1	64.5	0.24	conserved hypothetical Ustilaginaceae-specific protein
35	UH_08134	um05312	2.27E-43	45.8	74.8	0.38	conserved hypothetical Ustilaginaceae-specific protein
36	UH_10032	um04367	0	57.9	75.4	0.08	related to pol protein
37	UH_13915	No hit in Um					
38	UH_13916	um05318	2.01E-08	58.3	83.3	0.28	hypothetical protein
39	UH_10033	um05319	4.24E-13	36.7	65.1	0.28	conserved hypothetical Ustilaginaceae-specific protein
40	UH_08135	um10558	4.92E-300	93.2	96.8	0.93	probable tubulin beta chain
41	UH_08136	um02237	0	26.7	53.9	0.13	conserved hypothetical protein
42	UH_08137	um10560	0	49.1	73.3	0.4	conserved hypothetical protein
43	UH_08138	um10561	0	81.6	93.4	0.84	related to VPS10 domain-containing receptor SorCS1 precursor
44	UH_08139	um03753	8.65E-19	23.4	55.7	0.13	conserved hypothetical Ustilaginaceae-specific protein
45	UH_13921	No hit in Um					
46	UH_13922	um05325	0	64.9	78.4	0.66	conserved hypothetical protein

Number [1]	*U. hordei* MIPS ID [2]	*U. maydis* homolog [3]	ExpValue [4]	Percent Identity [5]	Percent Similarity [6]	score/selfscore ratio query [7]	Function [8]
47	UH_13925	um05326	0	54.3	72	0.5	conserved hypothetical protein

[1]Number corresponds to predicted genes in the figure.

[2] MIPS *Ustilago hordei* Database gene ID; The protein sequences of *U. hordei* strain 364 *(MAT-1)* were obtained from our collaborators and will be publicly available at http://mips.helmholtzmuenchen.de/genre/proj/MUHDB/ after the publication of the analysis.

[3]MIPS *Ustilago maydis* Database gene ID

[4]SIMAP results of the best hit; SIMAP is a program that measures protein similarity based on identities of amino acids in homologous fragments multiplied by the length of the homologous region and divided by the protein length (Rattei, et al. 2010)

[8]Function of *U. hordei* gene (query).

CHAPTER 3

Genome-wide analysis of *Ustilago hordei* candidate secreted effectors proteins; comparison with *U. maydis*[1]

3.1. Introduction

Pathogens secrete numerous effectors that play important roles in mediating infection of their hosts. Individual pathogen genomes encode dozens of secreted effectors that, in case of plant pathogens, are directed to the host apoplast, cytoplasm or nucleus depending on their target (Cunnac, et al. 2009, Jiang, et al. 2008, Kamoun 2006, Lindeberg, et al. 2009a, Lindeberg, et al. 2009b, Tyler, et al. 2006). As effector proteins interact with proteins in host cells and tissues, they are the direct targets of evolutionary forces and are expected to rapidly evolve. Most of the virulence and avirulence effectors from eukaryotic phytopathogens described to date are small secreted proteins (SSPs) (Rep 2005). It has been shown that these effector-encoding genes have orthologs in related species but due to an accelerated rate of evolution, they have diverged sequences compared to the rest of the genome (Liu, et al. 2011, Schirawski, et al. 2010). In *Phytophthora sojae* and *Phytophthora ramorum*, the RXLR effector-encoding genes have evolved at a faster pace compared to the rest of the genome (Jiang, et al. 2006). A three-genome comparison of *P. sojae, P. ramorum*, and *Phytophthora infestans* showed that effector families are evolving expeditiously in each species, apparently due to evolutionary pressure from their host plant (Tyler 2009). Only 25% of the effectors from these pathogens have orthologs in other species and less that 25% of these occur in a syntenic region, although the overall synteny is very high in these oomycete genomes (Tyler 2009, Tyler, et al. 2006). Raffaele et al. (2010) compared four genomes of closely related *Phytophthora* species that cause disease on different host plant species, in which they found that most of the pathogen genes and genome regions are highly conserved but the RXLR effectors are highly diverged.

[1] A version of this chapter will be included in a joint manuscript and submitted for publication: Laurie, J., Ali, S Linning, R., Bakkeren, G., Schirawski, J, Kahmann, R. *et al*.

Also, most of these genes are located in transposon-rich, gene-poor regions, which suggests a rapid evolution of effector loci after a change in the host (Raffaele, et al. 2010). The secreted effectors of *Blumeria graminis* f. sp. *hordei* (*Bgh*) which contain the conserved motif, YXC, are also very species specific and only 4% have orthologs in two other powdery mildew fungi that infect dicotyledonous plants (Spanu, et al. 2010).

U. hordei and *U. maydis* are two closely related basidiomycete phytopathogens, causing disease on two related cereal crops, barley and corn respectively, and having a similar life cycle leading to the infectious form (Hu, et al. 2002, Kamper, et al. 2006). As biotrophs, both *U. hordei* and *U. maydis* make a close association with the host and, analogous to other plant pathogens, likely secrete effectors into the host plant. The secretion of an effector in the host plant has been shown for at least one *U. maydis* effector, PEP1 (Basse, et al. 2000, Doehlemann, et al. 2009). In *U. maydis*, 554 protein-encoding genes have been predicted to be secreted and the expression of several of them has been shown to be induced during colonization of the host plant (Mueller, et al. 2008). More than 26% of these genes are clustered in seventeen clusters (Kamper, et al. 2006, Schirawski, et al. 2010).

The increasing number of complete genome sequences of related fungal and oomycete pathogens provides an opportunity to predict a complete set of secreted effector proteins for comparison. The genome of *U. hordei* has been sequenced by our laboratory in collaboration with J. Schirawski, Georg-August-Universität Göttingen, Germany, and R. Kahmann, Max Planck Institute for Terrestrial Microbiology, Marburg, Germany. The draft genome sequence of *U. hordei* strain 364 is approximately 26 Mb and encodes 7,113 predicted proteins (Laurie, J., Ali, S Linning, R., Bakkeren, G., Schirawski, J, Kahmann, R. *et al.,* in preparation). The predicted sets of effectors from fungi have an N-terminal SP and in some cases a non-conserved RXLR-like motif that could be involved in targeting these effectors to host cells (Dean, et al. 2005, Godfrey, et al. 2010, Kale, et al. 2010, Kamper, et al. 2006, Schirawski, et al. 2010). This N-terminal SP can be predicted by a number of computational tools to arrive at a comprehensive suite of potentially secreted proteins, tentatively called the "secretome" of a particular organism (Lee, et al. 2006a, Lee, et al. 2006b). In this chapter, I describe computational methods to arrive at a set of predicted secreted proteins from *U. hordei* to identify candidate secreted effector proteins (CSEPs) for screening for virulence or avirulence functions. In addition, I present a comparative analysis with the predicted set of *U. maydis* secreted proteins.

3.2. Materials and methods

3.2.1. Genomic resources

The genome and protein sequences of *U. hordei* strain 364 *(MAT-1)* were obtained from our collaborators and will be publicly available at http://mips.helmholtzmuenchen.de/genre/proj/MUHDB/ after the publication of the genome analysis. Sequences for *U. maydis* strain 521 *(a1b1)* were obtained from http://mips.helmholtz-muenchen.de/genre/proj/ustilago/.

3.2.2. Prediction of secreted proteins

A combination of several computational tools and software programs, SignalP 3.0, TMHMM 2.0, Target P 1.1 (http://www.cbs.dtu.dk/services/SignalP/ TMHMM/TargetP) and ProtComp 9.0 (http://linux1.softberry.com/berry.phtml) were used (Emanuelsson, et al. 2007) in a bioinformatic pipeline for the prediction of secreted proteins in *U. hordei*. All 7,113 predicted proteins were first analyzed by the SignalP 3.0 program to predict a SP based on SignalP-HMM results. This resulted in 1,142 SP-predicted proteins, which were subsequently run through TMHMM 2.0 to identify transmembrane proteins. Proteins with 1 transmembrane domain (TMD) were kept in the data set if the TMD was located close to the predicted signal peptide, since this could indicate a function related to translocation and not necessarily predict membrane retention (Mueller, et al. 2008). All other proteins with predicted transmembrane domains were removed. The resulting 931 proteins were subsequently screened by TargetP 1.1 to identify and remove proteins that were predicted to be mitochondrial; resulting in a set of 540 predicted secreted proteins. These 540 protein sequences were then analyzed with ProtComP 9.0 which compared them to proteins in the LocDB and PotLocDB databases, which hold proteins with data on known or reliably predicted localizations. Proteins with a predicted extra-cellular localization or with no similarity in the database were kept in the data set, resulting in 515 predicted secreted proteins.

3.2.3. Genome comparison

Predicted proteins from *U. hordei* strain 364 and *U. maydis* strain 521 were compared using Similarity Matrix of Proteins (SIMAP) at the Munich Institute for Protein Sequences (MIPS). SIMAP is a program that measures protein similarity based on identities of amino acids in homologous fragments multiplied by the length of the homologous region and divided by the protein length (Rattei, et al. 2010). After comparing all *U. hordei* proteins to all *U. maydis* proteins, they were divided into three groups on the basis of amino acid identities (SIMAP values; as described in Schirawski et al, 2010). Proteins with an amino acid identity of less than 20% were considered species-specific, proteins with values between 20 and 57% were grouped as moderately conserved, and above 57%, proteins were judged to be highly conserved between the two species. In addition, within the set of secreted proteins of *U. hordei*, I investigated manually whether the corresponding genes were arranged in clusters on the genome and then looked for possible synteny in *U. maydis.*

3.2.4. Phylogenetic analysis of *U. hordei* candidate secreted effectors proteins

All *U. hordei* proteins were run against one another at the MIPS using SIMAP to obtain a list of paralogs. Among the *U. hordei* CSEPs, proteins were considered true paralogs at a SIMAP value of 20% or greater. The phylogram of *U. hordei* CSEPs and their paralogs was constructed by using the multiple sequence alignment function in MEGA software, version 5 (Tamura, et al. 2011). A total of 495 proteins (372 CSEPs and 123 Non-Predicted Secreted Paralogs of CSEPs or NPSPCs) were used for sequence alignment. The resulting alignment was then used as input in MEGA version 5 to generate the neighbor-joining (NJ) tree using the Poisson correction method and number of amino acid substitution per site as the unit. The tree was subsequently depicted as a circular linearized tree. The main objective of the phylogram is to provide an overview of the effector diversity.

3.3. Results

3.3.1. Candidate predicted secreted proteins of *U. hordei*

U. hordei strain 364 has 515 predicted secreted proteins according the computational screening described in the Materials and Methods section, out of a total of 7,113 annotated ORFs. Previously, predicted secreted proteins in *U. maydis* had been further analyzed on the basis of similarity to known proteins with annotated functions such as enzymatic functions as to arrive at a set of CSEPs (Mueller, et al. 2008). In analogy, I subdivided the set of 515 predicted secreted proteins into two classes using annotations from MUHMB (a combination of computational analysis and manual curation). 157 of the proteins had a specific annotated function and the majority of them encoded secreted enzymes involved in cell wall modifications of plants, degradation of other plant components, modification of fungal cell walls and modification of metabolites. Fourteen of these annotated proteins are related to Mig (Maize induced gene) proteins that are induced during biotrophic growth in *U. maydis* and are considered effectors (Basse, et al. 2000). The remaining 358 predicted secreted *U. hordei* proteins are hypothetical, conserved hypothetical, or conserved hypothetical Ustilaginaceae-specific proteins that did not have any annotated function and could be potential effector proteins. After adding the 14 Mig proteins to the hypothetical predicted secreted proteins, the total number of CSEPs in *U. hordei* strain 364 is 372.

3.3.2. Phylogeny of CSEPs and their paralogs

I also searched the paralogs of the 372 *U. hordei* CSEPs and found 1,446 unique paralog proteins. At a SIMAP value of ≥20% identity, this number was reduced to 135 non-predicted secreted unique proteins. Twelve of these had annotated functions and were therefore removed from the candidate list, leaving 123 of these paralogs as hypothetical proteins. Adding these resulted in a total set of 495 proteins, 481 of which were accepted in the alignment algorithm (Material and Methods); the remaining proteins were discarded from the analysis because the pairwise distance could not be estimated. Interestingly, the 123 paralogs pulled into the set did not have any signal peptides and were therefore not included in the initial set based on SignalP and TargetP searches; however, by definition, they are related to the predicted effectors. An

example is the four additional Mig1-related proteins that have a similar cysteine pattern (Table 3.1). Such non-predicted secreted paralogs of CSEPs (NPSPCs) expand phylogenetic groups resulting in greater sequence diversity. Overall, many NPSPCs grouped with various CSEP protein families (Fig 3.1).

3.3.3. Cysteine- rich secreted protein

A number of pathogen effector molecules, among which are proven avirulence proteins, have specific folding requirements: secondary and tertiary structures that often require the presence of disulphide bonds which help in protein stability and function (Doehlemann, et al. 2009, Joosten, et al. 1994, Müller, et al. 2008, Rooney, et al. 2005, Stergiopoulos, et al. 2010, Teertstra, et al. 2006, Tian, et al. 2008, Tian, et al. 2004, van den Burg, et al. 2006). Disulphide bonds or 'bridges' formed between cysteine residues. Several examples exist of avirulence proteins having mutations in a virulence allele where the cysteine residue has been replaced resulting in a changed conformation and, consequently, a loss of function (Joosten, et al. 1994, Doehlemann et al 2009). The 372 *U. hordei* CSEPs and their 123 paralogs with SIMAP value of ≥20% were, therefore, analyzed for their cysteine residue content. This analysis revealed different numbers of cysteine residues, varying from zero to as many as 28. Of the 372 CSEPs, 190 had four or more cysteine residues potentially involved in disulphide bridge formation, while this number was 90 among the 123 paralogs. Among these cysteine-rich effectors, I identified 71 proteins which could be tentatively placed in 20 classes based on their characteristic spacing of the cysteine residues (Table 3.1).

3.3.4. Comparison of *U. hordei* and *U. maydis* secretomes

Based on the similarity criteria outlined in the Materials and Methods section, a comparison to the *U. maydis* protein complement categorized 16.9% (1,203) of the total proteins as *U. hordei*-specific (having amino acid identities, i.e., SIMAP values, of less than 20%); out of these, 11.6 % (140 proteins) are predicted to be secreted. In contrast, 83.1% of *U. hordei* proteins (5,910) have homologs in *U. maydis* (having amino acid identities, i.e., SIMAP values, of 20-100%), whereas only 6% of these are predicted to be secreted. Among the homologous proteins, 15% (1,067) fall in the moderately conserved group with 10.9% predicted to be secreted, while 68%

(4,837) are in the highly-conserved group in which only 5.5% are predicted to be secreted. As a general observation, more predicted secreted proteins are found in the species-specific and less-conserved "homologous" proteins (Fig 3.2). 1,203 proteins are present only in *U. hordei*, while 631 exist only in *U. maydis*. Among predicted secreted proteins, 355 *U. hordei* proteins, out of the 495, have orthologs to, and are more or less conserved with, *U. maydis* proteins, while 140 proteins seem *U. hordei*-specific.

3.3.5. A subset of predicted secreted proteins and their paralogs reside in clusters

Many of the predicted secreted effector proteins in *U. maydis* (Kamper, et al. 2006) and *Sporisorium reilianum* (Schirawski, et al. 2010) are arranged in clusters. I analyzed the *U. hordei* genome for the localization and arrangement of the 372 CSEPs and 123 NPSPCs and found at least 62 clusters containing a subset of these predicted effector proteins and their paralogs. Clusters were defined as regions of the genome that contained at least two consecutive CSEPs and NPSPCs or were interrupted by only a few non-related proteins. The following findings substantiated the cluster definition: the 62 *U. hordei* clusters contained 389 genes and more than 51% of these genes encoded CSEPs or NPSPCs, while overall only 7% of the total *U. hordei* genes encoded CSEPs and NPSPCs. These clusters contained from two to as many as 32 CSEPs and NPSPCs. There are 199 CSEPs and NPSPCs in the 62 identified clusters accounting for approximately 40% of the total CSEPs and NPSPCs in *U. hordei*.

3.3.6. Comparison between the *U. hordei* and *U. maydis* secreted protein clusters

Among the 62 identified *U. hordei* clusters, 9 had predicted secreted proteins that had highly-conserved homologs in *U. maydis* (SIMAP value higher than 57%), whereas 39 had predicted secreted proteins that were "least-conserved" or species-specific (SIMAP value between 20% and 57%, or 20% and below, respectively);14 clusters harboured both of those last two classes. In *U. maydis*, seventeen of the clusters, accounting for more than 26% of the predicted secreted proteins in that species, had been deleted individually. Deletion of either of six clusters reduced disease, while for two clusters, disease ratings on corn seedlings increased; it was concluded that many of the effectors residing in these clusters had a function in virulence (Kamper, et al. 2006, Schirawski, et al. 2010). Ten of these seventeen investigated *U. maydis* clusters were conserved

in *U. hordei* although two of those ten *U. maydis* clusters (e.g. 3A and 9A; Kamper, et al. 2006) were not identified in *U. hordei* genome analysis using the criteria for effectors in this study because they had annotated functions; these were discarded from further analysis. Among the remaining eight syntenic clusters, four contained *U. hordei* CSEPs and NPSPCs that are least conserved in an otherwise highly-conserved, syntenic region and the deletion of the homologous *U. maydis* clusters either increased or decreased pathogenicity. The remaining four syntenic clusters contained conserved homologs for almost all CSEPs in both *U. hordei* and *U. maydis* and the deletion of these clusters in *U. maydis* did not have any effect on pathogencity (Kamper, et al. 2006, Schirawski, et al. 2010). There seems to be a strong correlation between the degree of conservation among homologous effectors between the fungal species and the effect they have on virulence. The less-conserved, more-diverged effectors seem to have a more-defined role in virulence.

3.4. Discussion

The sequencing of plant pathogen genomes provides an opportunity for pathologists to identify all predicted secreted proteins of pathogens and develop hypotheses regarding their potential interaction(s) with host plants. Here, I used a bioinformatic approach to identify a set of predicted secreted proteins (the secretome) from the recently sequenced genome of the barley smut pathogenic fungus, *U. hordei* (Laurie, J., Ali, S Linning, R., Bakkeren, G., Schirawski, J, Kahmann, R. *et al* in preparation). Among these, I expect to find many candidates effector genes with virulence or avirulence functions. I also determined whether these effectors are clustered and compared the secretomes of *U. hordei* and the closely-related corn smut fungi, *U. maydis* and *S. reilianum*.

In *U. maydis*, there are 386 predicted secreted proteins that lack any annotated enzymatic function (Mueller, et al. 2008) and this is similar to the number of effectors I identified in *U. hordei*. It has been shown that effector PEP1 from *U. maydis*, which is required for penetration of the host plant, is conserved in *U. hordei* and is a functional ortholog since it can complement a *Pep1*-deficient *U. maydis* mutant (Doehlemann, et al. 2009). These two fungi are closely related; 84% of the *U. hordei* genes have orthologs in *U. maydis* and 90% of *U. maydis* genes have orthologs in *U. hordei*; however, there is a difference in conservation among secreted and non-secreted proteins between the two species. The majority of the predicted secreted *U. hordei*

effectors are either species-specific or fall into the `least conserved` category of genes in *U. maydis* (Fig 3.2). Only 31% of the predicted *U. hordei* effectors are highly conserved in *U. maydis*, which is very low compared to 68% of all proteins which are highly conserved between *U. hordei* and *U. maydis*. This suggests that the effectors are evolving faster than the rest of the genome, most likely because of the selection pressure they face from interactions with their respective host plants. This trend of higher evolution among effectors has been shown for several other plant pathogens. In three closely related oomycetes, *P. sojae, P. ramorum*, and *P. infestans*, which show very high overall synteny in their genomes, only 25% of the effectors have orthologs in other species and more than 75% of these are not in syntenic regions (Tyler 2009, Tyler, et al. 2006). Similarly, only 4% of the secreted effectors of *B. graminis* f. sp. *hordei* that contain the conserved motif, YXC, have orthologs in two other powdery mildew fungi infecting dicotyledonous plants (Spanu, et al. 2010). The different degree of conservation among the CSEPs in *U. hordei* and in *U. maydis* may be related to their function. The effectors that are highly conserved between the two species may have some basic role in causing infection on plants. On the other hand, the species-specific effectors probably evolved more recently because of the arms race between the pathogen and its specific host plant. The number of predicted secreted effectors in smut fungi are relatively small compared to other fungal pathogens such as the poplar leaf rust *Melampsora larici-populina* and the wheat steam rust *Puccinia graminis* f.sp *tritici* and ascomycetes such as *M. oryzae* (Duplessis, et al. 2011, Yoshida, et al. 2009). In *M. larici-populina*, a set of 1,184 predicted SSPs has been identified, 74% of which are species-specific while 85% of 1,103 predicted SSPs are species-specific in *P graminis* f.sp *tritici* (Duplessis, et al. 2011). In *M. oryzae*, 1,206 putative secreted proteins have been identified (Yoshida, et al. 2009). In obligate biotroph *B. graminis* f. sp. *hordei*, 248 predicted secreted proteins have been identified that did not have any homologs outside the group of mildew fungi analyzed (Spanu, et al. 2010). However, one should keep in mind that different groups may be using slightly different approaches for identification of predicted secreted proteins.

105 CSEPs (28%) have at least one paralog in the *U. hordei* genome not predicted to be secreted, i.e., have no SP identified. Moreover, some of the CSEPs have paralogs that group together in gene families with non-predicted secreted proteins. These paralogs may have either lost their signal peptide during recombination and duplication, or they may still be secreted through a non-classical secretion pathway. Gene families generally arise by duplication of some

part of the chromosome and one copy can keep the original function while others can undergo mutations and can get a diverged function. Several extracellular effectors from *Phytophthora* species are encoded by multigene families, and some of these families have orthologs in other plant pathogens, like bacteria, while some are *Phytophthora*-specific (Dong, et al. 2009, Liu, et al. 2005, Qutob, et al. 2009, Torto, et al. 2003).

More than 50% (190) of the *U. hordei* CSEPs, have four or more cysteine residues. In several fungal pathogens that colonize the apoplastic space, secreted cysteine-rich effectors have been found; disulphide bridge formation serves as a mechanism to protect them from apoplastic proteases (Kamoun 2006b, Rep 2005, Tian, et al. 2004, Tian and Kamoun 2005, Tian, et al. 2007, van Esse, et al. 2007, van Esse, et al. 2008). This number of cysteine-rich effector proteins is higher for intracellular biotropic pathogens that make a close association with host cells, but the disulphide bond may be required for structure and function rather than protection from apoplastic enzymes in biotrophs (Catanzariti, et al. 2007). In biotrophic fungus, *M. lacaria-populina* that parasitizes poplar, a large number of potential effectors have been identified to be cysteine-rich (Duplessis, et al. 2011). In *U. hordei*, several of the cysteine-rich proteins were grouped into twenty classes of effector proteins based on characteristic cysteine content and spacing (Table 3.1). The length of the proteins and the number of cysteine residues is not the same among these classes. Class I contains Mig1-related and Mig-like proteins that have been shown to be induced in *U. maydis* during maize infection (Basse, et al. 2000, Basse, et al. 2002). Although a *Mig1* deletion mutant did not affect pathogenicity of *U. maydis* on corn (Basse, et al. 2000), the deletion of the whole cluster of *Mig1* paralogs increased virulence on corn (Schirawski, et al. 2010). It was suggested that this *Mig1* cluster might contain an avirulence effector because of its conserved similarity to some other fungal avirulence proteins. In *U. maydis* there are four Mig1 effectors, while in *S. reilianum* there are ten (Schirawski, et al. 2010). This group with characteristic cysteine spacing is represented by 18 effectors in *U. hordei*; 13 of these are predicted to be secreted while five are not because no SPs can be identified. The SP-encoding sequences may have been lost during duplication by recombination in these genes. Interestingly, in *U. maydis* and *S. reilianum*, these *Mig1* genes are clustered together, while in *U. hordei*, there are two clusters each with two genes and the rest are dispersed and reside at 14 different loci. Class II cysteine-rich effectors contain Mig2-like proteins with similar characteristic cysteine spacing, while one member, UH_14902, has one cysteine residue

less and is 60 amino acid shorter than the other two members of the class. The remaining 18 classes all contain hypothetical secreted proteins with no known functions. In several fungal and oomycete pathogens, it has been shown that these cysteine-rich apoplastic effectors are inhibitors of host hydrolytic enzymes, protecting the pathogen from their devastating effect, or they are protectors that bind to fungal chitin and provide a shield (Rooney, et al. 2005, Tian, et al. 2004, Tian, et al. 2007, Shabab, et al. 2008, van den Burg, et al. 2006, van Esse, et al. 2008).

To assess whether the predicted secreted proteins were encoded by genes residing in clusters, CSEP genes were located on the *U. hordei* genome and the flanking regions were searched manually. The majority of the genes in these clusters encoded hypothetical, conserved hypothetical or *Ustilaginaceae*-specific conserved hypothetical proteins. They were very similar to gene clusters in *U. maydis* and *S. reilianum* (Kamper, et al. 2006, Schirawski, et al. 2010). Schirawski et al (2010) performed a comparative genomic study of *U. maydis* and *S. reilianum* and identified 43 diverged gene clusters in which 94% of the genes encoded hypothetical proteins and 61% predicted secreted proteins. In Chapter 2, I discussed one such related cluster and the involvement of at least one member, *UhAvr1*, in gene-for-gene interactions in *U. hordei*. In this manner, the *U. hordei* clusters are different from the *U. maydis* and *S. reilianum* clusters of secreted proteins, as no such avirulence function has been identified in these two fungi with corn so far.

This type of clusters in these Ustilaginomycete fungi is unique among fungi; no such arrangement has been identified in other completed fungal genomes such as *M. oryzae* (Dean, et al. 2005) and *B. graminis* f. sp. *hordei* (Spanu, et al. 2010). Gene clusters in other fungi are usually involved in sexual reproduction, biosynthesis, or degradation of secondary metabolites (Gardiner, et al. 2004, Kupfahl, et al. 2006). In several ascomycetes such as *Fusarium solani*, *F. oxysporum*, *Alternaria alternata* and *Cochliobolus* spp., genes for putative virulence functions are located on the same chromosome close to one another and sometimes interspersed with repeats, transposable elements, or other genes (van der Does and Rep 2007). In *Leptosphaeria maculans*, avirulence genes are clustered in a large region full of retrotransposons and their remnants, interrupted by housekeeping genes (Fudal, et al. 2007, Gout, et al. 2006). It is not understood how genes involved in virulence or avirulence evolve to cluster in fungal pathogens. Van der Does and Rep (2007) proposed two possible mechanisms for the evolution of virulence gene clusters in fugal pathogens. According to the first hypothesis, virulence genes may appear

at random positions in the genome and then cluster together as a result of random gene shuffling and a selection force driving clustering as a strong selective advantage. Clustering may be necessary for co-regulation of the genes, especially if several virulence genes are required for infection of a particular plant host. The clustering of the virulence genes or at least the co-location on the same chromosome will be necessary for genes that are horizontally transferred from a pathogen to a non-pathogen or a different lineage of fungi. In *F. oxysporum* f. sp. *lycopersici*, there are at least six avirulence genes on lineage-specific chromosome 14 that are considered to have been gained through horizontal gene transfer (Ma, et al. 2010). Similarly, it has been shown for *M. oryzae* that at least 316 candidate effectors are present on a 1.68 Mb-region in strain Ina168 but absent in the sequenced isolate, 70-15 (Yoshida, et al. 2009). The second hypothesis is that some parts of the genome are more receptive to the emergence of new genes or virulence gene insertion. It may be the reason that several effectors encoding genes are located close to repetitive elements and transposable elements that may be involved in their accumulation (Fudal, et al. 2005, Fudal, et al. 2007, Gout, et al. 2006, Kang, et al. 2001, Khang, et al. 2008, Zhou, et al. 2007). Transposons are also involved in chromosome rearrangements and might assist the insertion of new genes or chimeric gene synthesis as a result of recombination (Thon, et al. 2006, van der Does and Rep 2007, Wostemeyer and Kreibich 2002).

I described here a genome-wide, computational prediction and analysis of CSEPs in *U. hordei* and a comparison of that set with a similar set in the closely related corn smut fungi, *U. maydis* and *S. reilianum*. Having identified these CSEPs, it will first be necessary to show that these effectors are actually secreted, which may be possible using the Yeast Secretion Trap system (Lee, et al. 2006). Transcriptome profiling using *U. hordei* microarrays or RNA sequencing could be used to look for effectors that are up-regulated during biotrophic growth to reduce the number of CSEPs further for experimental approaches. The functional analysis of these effectors could involve transient expression in different barley cultivars using Agroinfiltration in order to identify effectors that have an avirulence function. Interaction of such an avirulence effector and a matching resistance gene could result in a HR response; however, current investigated incompatible interactions between *U. hordei* and barley normally do not result in a macroscopically visible HR response (Chapter 2). An alternative approach could be co-infiltration of effector genes together with marker genes such as *GUS* or *GFP*; an avirulence function will result in cell death and consequently in a decrease in marker gene expression

(Dong, et al. 2009, Rehmany, et al. 2005). To look for any virulence function of these CSEPs, such as suppression of host defense responses, Agroinfiltration of these effectors followed by inoculation of a secondary barley pathogen such as *B. graminis* f. sp. *hordei* will be helpful. One bigger challenge will be to demonstrate the site of action of these effectors, i.e. to determine whether they function in the apoplast to disarm a plant defense enzyme or can enter the host cytoplasm or even the nucleus and have a function there to suppress host defense and establish compatibility. Chimeric constructs with fluorescent proteins and confocal microscopy could possibly be used for such an approach.

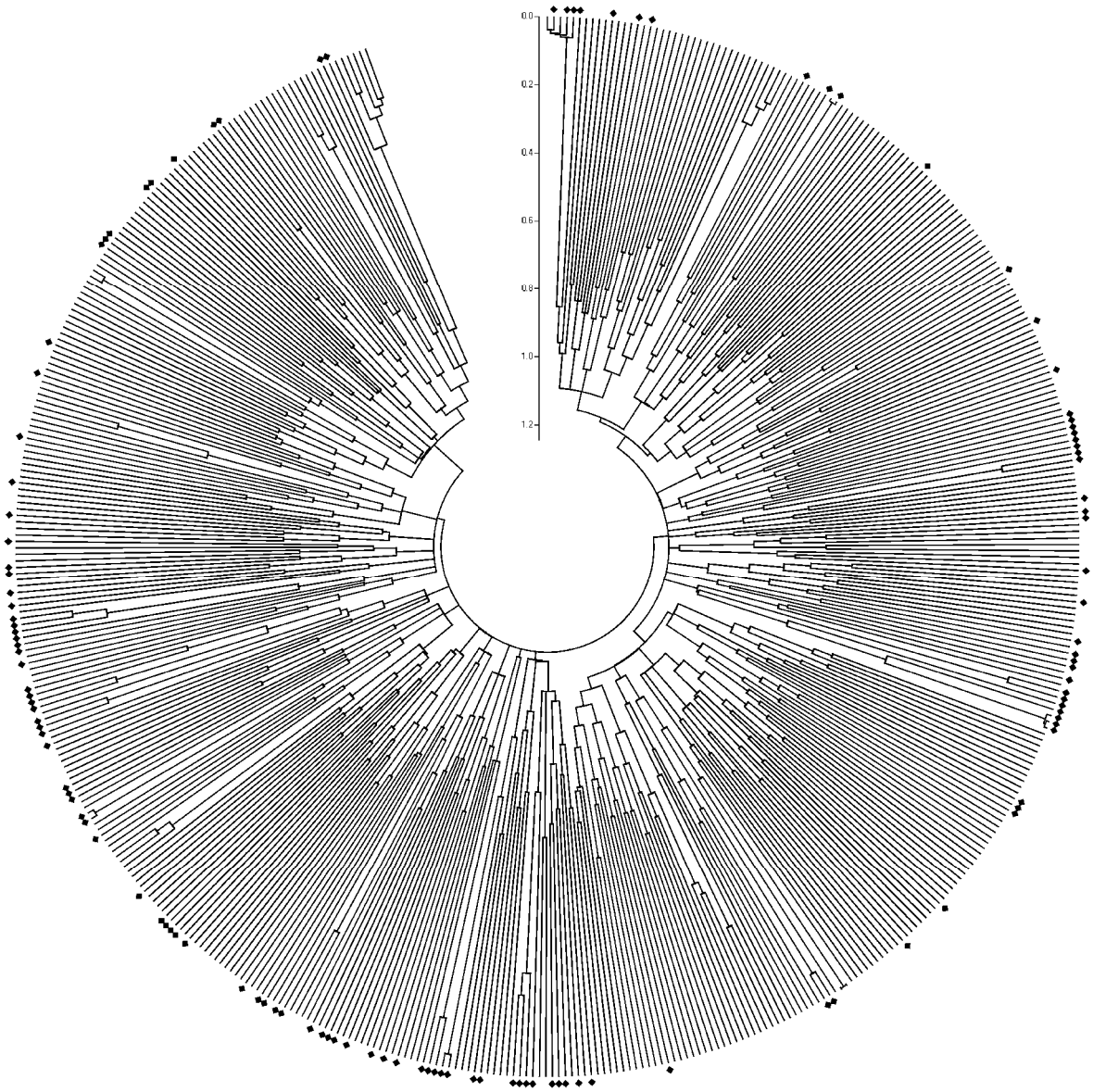

Figure 3.1 Overview of the molecular relatedness between *U. hordei* CSEPs and NPCSEPs showing a large diversity and small families. Circular neighbor-joining phylogenetic tree of 481 amino acid sequences (372 CSEPs and 123 non-predicted secreted paralogs, the latter marked by diamond shapes). Fourteen sequences whose pair-wise distance could not estimated, were discarded from the analysis. The tree is drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site.

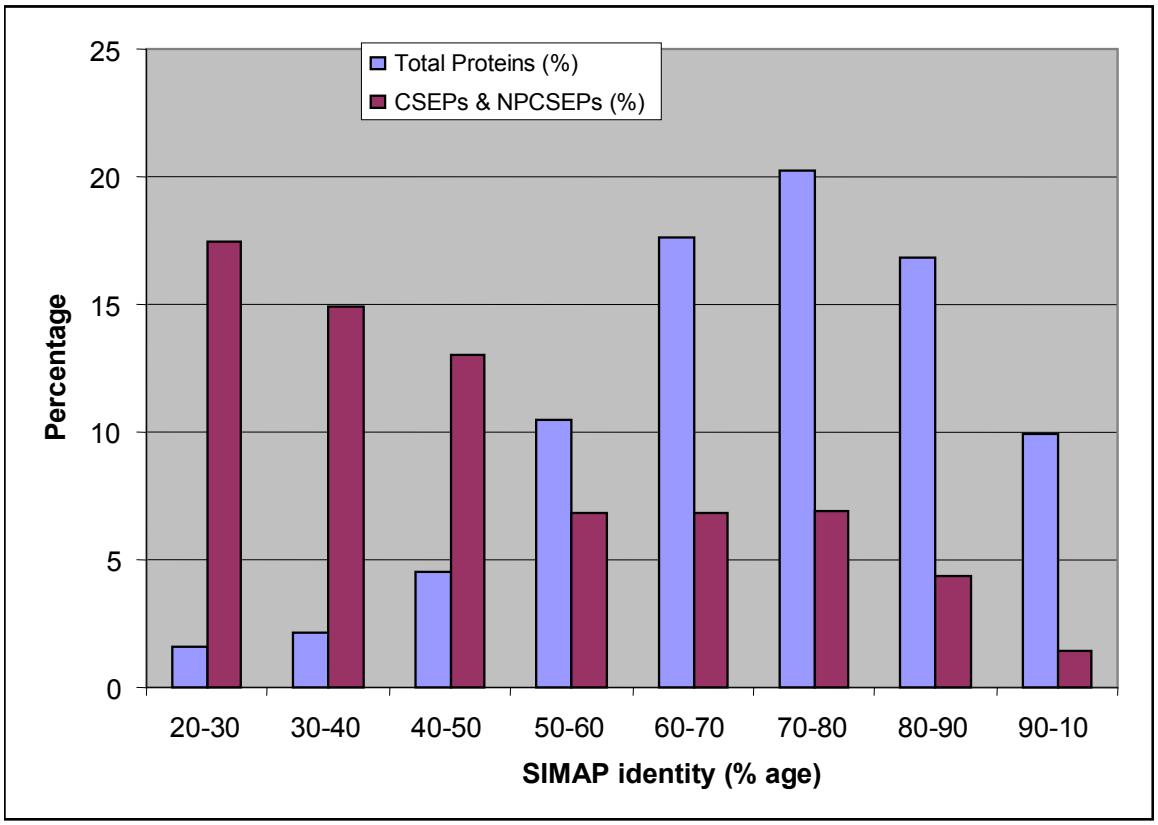

Figure 3.2 Diversity among *U. hordei* and *U. maydis* proteins. Depicted is the distribution of amino acid identities in 10% increments (X-axis) among all *U. hordei* and *U. maydis* proteins (blue bars) as a percent of the total complement of 7,113 *U. hordei* proteins (Y-axis). The red bars represent the distribution of amino acid identities compared to *U. maydis* homologs of the predicted *U. hordei* CSEPs and NPCSEPs as a percentage of the 495 predicted SSPs.

Table 3.1 *U. hordei* candidate secreted effectors proteins with characteristic patterns of occurring cysteine residues (C) and spacing (number of X amino acid residues)

Class	Protein ID	Location of cysteine residue and spacing
I	UH_06051	C-X14-C-X9-C-X13-C-X53-C-X39-C
	UH_06234	C-X14-C-X9-C-X13-C-X53-C-X39-C
	UH_04736	C-X14-C-X9-C-X13-C-X52-C-X39-C
	UH_08826	C-X14-C-X9-C-X13-C-X53-C-X39-C
	UH_06702	C-X14-C-X9-C-X13-C-X53-C-X39-C
	UH_05040*	C-X3-C-X12-C-X52-C-X39-C
	UH_12818*	C-X39-C
	UH_13038*	C-X39-C
	UH_16113*	C-X39-C
	UH_08403*	C-X2-C-X9-C-X31-C-X2 -C-X9 -C-X14-C-X2 -C-X9 -C-X14-C-X2-C-X9-C
	UH_15214	C-X62-C-X15-C-X6 -C-X68-C-X42-C-X21
	UH_04923	C-X11-C-X31-C-X15-C-X9 -C-X67-C-X37-C
	UH_08252	C-X40-C-X15-C-X9 -C-X50-C-X8 -C-X39-C
	UH_04676	C-X16-C-X26-C-X15-C-X9 -C-X66- C-X37-C
	UH_04675	C-X11-C-X4- C-X16-C-X14-C-X66- C-X39-C
	UH_04922	C-X16-C-X27-C-X15-C-X9- C-X42-C-X9 -C-X37-C-X37-C
	UH_04990	C-X17-C-X9 -C-X65-C-X41-C
	UH_06803	C-X17-C-X9 -C-X69-C-X41-C
II	UH_14902	C-X34-C-X9-C-X21-C-X2-C-X12-C
	UH_08758	C-X45-C-X35-C-X9-C-X21-C-X2-C-X12-C
	UH_12644	C-X47-C-X34-C-X9-C-X21-C-X2-C-X12-C
III	UH_05003	C-X22-C-X20-C-X2-C
	UH_01593	C-X22-C-X20-C-X2-C
	UH_07232	C-X22-C-X21-C-X2-C
	UH_01836	C-X23-C-X22-C-X2-C-X123-C-X27-C

Class	Protein ID	Location of cysteine residue and spacing
IV	UH_02419	C-X15-C-X29-C-X5-C-X15-C-X28-C-X8-C-X77-C-X2-C-X40-C
	UH_03994	C-X15-C-X30-C-X6-C-X15-C-X30-C-X8-C-X45-C-X2-C-X24-C
V	UH_08738	C-X21-C-X88-C-X3-C-X12-C-X8-C-X15-C-X59-C
	UH_05606	C-X21-C-X88-C-X3-C-X12-C-X8-C-X15-C-X59-C
VI	UH_03442	C-X50-C-X195-C-X54-C
	UH_03402	C-X12-C-X163-C-X14-C-X166-C-X54-C
VII	UH_00093	C-X10-C-X12-C-X80-C-X42-C-X16-C-X19-C-X13-C-X40-C-X14-C
	UH_00478	C-X10-C-X11-C-X20-C-X92-C-X3 -C-X8 -C-X8 -C-X15-C-X34-C
VIII	UH_14977	C-X6 -C-X6-C-X6 -C-X28-C-X9 -C-X5-C-X10-C
	UH_16458	C-X6 -C-X6-C-X6 -C-X28-C-X9 -C-X5-C-X10-C
	UH_01705	C-X14-C-X6-C-X44-C-X19-C-X37-C
IX	UH_14136	C-X4- C-X59-C-X8-C-X29-C-X3-C-X12-C-X17-C-X3-C-X4-C-X2-C
	UH_03910	C-X4-C-X2-C-X55-C-X8-C-X29-C-X3-C
	UH_04742	C-X4- C-X54-C-X8-C
X	UH_02707	C-X50-C-X6-C-X52-C-X4-C-X29-C
	UH_04984	C-X42-C-X6-C-X39-C-X4-C-X10-C-X40-C
XI	UH_08916	C-X17-CC-X17-C-X17-CC
	UH_08915	C-X24 -C-X17-CC-X17-C-X17-CC
	UH_07874	C-X1-C-X23-CC-X17-C
	UH_12082	C-X1-C-X11- C-X9- C -X47-C-X7 -C
	UH_14135	C-X6-C-X11- C-X11-C -X44-C
XII	UH_06080	CC-X59-C-X17-C-X10-C

Class	Protein ID	Location of cysteine residue and spacing
	UH_04531	C-X5- C-X17-C-X10-C-X13-C-X12-C-X1-C-X6-C
XIII	UH_06316	CC-X11- C-X10-C-X3 -C C-X3 -CC
	UH_14081	C-X4-C-X3-C-X10-C-X3 -C-X4 -C-X3 -C
	UH_02007	C-X3 -C-X12-C-X62-C-X21-C-X10-C-X4-C
	UH_00491	C-X4-C-X4-C-X4 -C-X4 -C-X56-C-X18-C-X24-C
	UH_05093	C-X4-C-X5-C-X6-C-X1-C-X7 -C-X33-C-X5 -C
XIV	UH_04400	C-X6-C-X3-C-X4-C-X22-C-X5 -C-X9 -C-X18-C-X40-C-X3-C
	UH_04949	C-X6-C-X7-C-X1-C-X53-C-X50-C-X63-C
XV	UH_14114	C-X3-C-X15-C-X108-C-X33-C-X157-C-X16 -C-X9 -C-X6-C
	UH_14108	C-X3-C-X15-C-X108-C-X33-C-X52 -C-X104-C-X31-C
XVI	UH_01209	C-X6-C-X20-C-X29-C-X6-C-X3-C-X21-C-X5-C-X9-C
	UH_01211	C-X6-C-X20-C-X30-C-X6-C
XVII	UH_01635	C-X6-C-X4-C-X10 -C-X8 -C-X1 -C-X18-C-X6 -C-X3 -C-X12 -C-X3 -C-X7-C
	UH_04865	C-X6-C-X4-C-X6 -C-X27 -C-X9 -C-X5 -C-X12 -C-X75-C-X6 -C-X5 -C-X6-C-X27-C-X9-C-X5-C-X8-C-X186-C
	UH_00039	C-X5-C-X7-C-X109-C-X78 -C-X75-C-X39-C-X158-C-X9 -C-X180-C-X29-C
	UH_08589	C-X5-C-X7-C-X27 -C-X124-C-X5 -C-X22-C-X26 -C-X7 -C-X12 -C
	UH_03101	C-X5-C-X4-C-X4 -C-X12 -C-X5 -C-X5 -C-X4 -C-X9 -C-X5 -C-X4 -C-X4-C-X11-C-X5-C-X5-C-X4-C-X9-C -X5-C-X4-C-X4-C-X11-C-X5- C-X4-C-X4-C-X9-C-X5-C-X4-C-X5-C
	UH_14724	C-X6-C-X5-C-X5 -C
XVIII	UH_01807	C-X8-C-X15-C-X169-C-X4 -C-X71-C-X79-C
	UH_01944	C-X8-C-X15-C-X60 -C-X19-C

Class	Protein ID	Location of cysteine residue and spacing
XIX	UH_00393	C-X27-C-X20-C-X6-C
	UH_01539	C-X2- C-X29-C-X6-C
XX	UH_01947	C-X11-C-X62-C-X19-C
	UH_01952	C-X13-C-X57-C-X18-C

* Proteins representing the non-predicted secreted protein paralogs of CSEPs.

CHAPTER 4

Introduction of large DNA inserts into the barley pathogenic fungus, *Ustilago hordei*, via recombined binary BAC vectors and *Agrobacterium*-mediated transformation[1]

4.1. Introduction

An understanding of cell development and pathogenicity in *U. hordei* will require an efficient genetic transformation procedure for gene complementation, gene replacement and other genome manipulations. It is also desirable to transfer large DNA fragments containing complete genes with regulatory sequences necessary for gene function, or complete clusters of genes, to assess the location of specific functions/genes on genome-size fragments as represented by Bacterial Artificial Chromosome (BAC) inserts (Shizuya, et al. 1992). For example, *U. hordei* harbours gene clusters coding for related predicted secreted proteins that could be effectors during the interaction with its host, similar to the clusters described in *U. maydis* (Kamper, et al. 2006).

There is no efficient and reproducible transformation system for *U. hordei*. Current methods use partial protoplasts and the addition of 1% polyethylene glycol (PEG) followed by electroporation (Bakkeren, unpublished). The generation of partial protoplasts involves the use of lytic enzymes, which have to be calibrated for each enzyme batch rendering this method not very reproducible. *Agrobacterium tumefaciens* is a well known plant pathogen causing crown gall disease on plants by transferring a part of its tumor inducing (Ti) plasmid DNA to plant cells and integrating this stably into the host genome. Any DNA between specific 25 base-pair imperfect repeats, termed left and right border sequences, can be transferred. Such constructs can be located on smaller replicating plasmids, so-called binary vectors (Hoekema, et al. 1983, Lee and Gelvin 2008). Commonplace in plant biotechnology as the agent of choice for genetic transformation, the *Agrobacterium*-mediated transformation (AMT) system

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has been exploited extensively for fungal transformation as well (Amey, et al. 2002, de Groot, et al. 1998, Michielse, et al. 2005a, Michielse, et al. 2005b, Sugui, et al. 2005, Tucker and Orbach 2007). Compared to conventional transformation methods, AMT is very efficient for many filamentous fungi and has worked well for the development of transformation systems for fungi which are refractory to transformation using conventional methods, such as *Agaricus bisporus*, *Aspergillus giganteus* and *Helminthosporium turcicum* (Chen, et al. 2000, Degefu and Hanif 2003, Meyer, et al. 2003, Mikosch, et al. 2001, Michielse, et al. 2005b). Among basidiomycetes, AMT is very efficient in *Cryptococcus neoformans* and *Cryptococcus gattii* (McClelland, et al. 2005) and *U. maydis* (Ji, et al. 2010) and was recently also used successfully for the genetic transformation of the flax rust, *Melampsora lini* (Lawrence, et al. 2010). AMT is not only more efficient compared to conventional methods but often results in single copy integration events at random sites in the fungal genome which is desirable for creating insertion mutations (Combier, et al. 2003, Mullins, et al. 2001, Takahara, et al. 2004). An additional advantage of AMT, compared to classical mutagenesis is the ease with which fungal sequences flanking the T-DNA insertion site can be recovered and identified (Bundock and Hooykaas 1996, Bundock, et al. 2002, de Groot, et al. 1998, Leclerque, et al. 2004). For example, sporulation deficient mutants, pathogenicity deficient mutants, antibiotic deficient mutant and mutants altered in pigmentation have been obtained in several fungi (Blaise, et al. 2007, Li, et al. 2005, Rogers, et al. 2004).

Conventional cloning methods for the generation of transformation constructs using restriction enzymes are often inefficient and time consuming, especially for BAC clones because of their large sizes and few convenient restriction sites (Nagano, et al. 2007). Recombineering as an alternative method uses the DNA double-strand repair machinery and bacteriophage lambda RED recombination proteins (Lee, et al. 2001) and does not require restriction sites for conventional cloning or specific recombination sites such as the ones required for 'Gateway™' cloning. Recombineering is therefore useful for cloning large DNA fragments and facilitates the cloning of whole or specific regions of BAC or PAC clones (Lee, et al. 2001, Raymond, et al. 2002).

Methods to transfer large genomic fragments in BAC clones to plants using AMT have been established many years ago when *Agrobacterium*-specific binary BAC vectors, so-called BIBAC vectors, were developed (Hamilton, et al. 1996). Combining above-mentioned techniques, Takken et al. (2004) developed a strategy to convert BAC vectors into fungal-

specific BIBAC vectors suitable for *Agrobacterium*-mediated transfer into fungal strains. This was efficiently achieved by a one-step procedure making use of *in vivo* recombineering (Lee, et al. 2001) of a linear 'fungal- and binary-specific' fragment into BAC clones.

In the present study I investigated the feasibility of using this system in *U. hordei* and testing whether large DNA fragments could be delivered stably into its genome via AMT. I converted two BAC clones containing genomic inserts into BIBACs and showed that *A. tumefaciens* can deliver these genomic fragments stably into the genome of *U. hordei*.

4.2. Materials and methods

4.2.1. Strains and plasmid

E. coli strain SW102, a recombineering strain derived from strain DY380 (Warming, et al. 2005), was obtained from Dr. N. Copeland (National Cancer Institute, Frederick, MD). Supervirulent *Agrobacterium* strain COR309 is a recA-deficient C58 nopaline strain UIA143 harbouring disarmed pTiB6 derivative plasmid pMOG101 (Hamilton, et al. 1996) and a special vir helper plasmid pCH32 which provides extra copies of the *virA* and *virG* two-component signaling genes. *Agrobacterium* strain COR308 is similar to COR309 except that it has disarmed pTi derivative plasmid pMP90 instead of pMOG101; they were obtained from Cornell University (http://www.biotech.cornell.edu/BIBAC/BIBACHomePage.html). *Ustilago hordei* haploid strain Uh4857-4 (alias Uh364, *MAT-1*) has been described (Linning et al. 2004) and *U. maydis* haploid strain 324 (*a2b2*) is identical to Um521 (Kronstad and Leong 1989). The REC plasmid pFT41 was obtained from Dr. F. Takken (Swammerdam Institute for Life Sciences, University of Amsterdam, The Netherlands; Takken, et al. 2004). pUSBAC5 is a BAC vector derivative of pEcBAC1 (Frijters, et al. 1997) converted for use in *Ustilago* species by introducing a specific hygromycin B resistance cassette (Linning et al. 2004).

4.2.2. Recombineering

Target constructs pUSBAC5, pUSBAC5_2-1 and pUsBAC5_1-6 were transformed into recombineering *E. coli* strain SW102, selected on Luria-Bertani (LB) plates supplemented with chloramphenicol (Cm) 20 μg ml^{-1} at 30 ^{o}C to prevent premature induction of the phage

recombineering genes (RED gene). Details of the protocol can be found in Lee et al. (2001). The recombining REC part was amplified from pFT41 by Polymerase Chain Reaction (PCR) with primers cat-f2 (CCGTTGATATATCCCAATGGC) and catR (ACAAACGGCATGATGAACCT) using TaKaRa LA Taq^{Tm} polymerase (TAKARA Bio INC) and the following program on a MyCycler (BioRad): an initial denaturation step of 5 min at 95 ^{o}C followed by 35 cycles of 30 sec at 95 ^{o}C, 40 sec at 58 ^{o}C and 10 min at 68 ^{o}C, with a final extension at 68 ^{o}C for 15 min. The PCR product was digested with DpnI to remove the template and then purified on an agarose gel using the QIAquick Gel extraction kit (QIAGEN) according to manufacturer's instruction. The SW102 cells harbouring the target BAC vectors were grown in LB medium supplemented with 20 µg Cm ml^{-1} at 30 ^{o}C to an optical density at 600 nm (OD600) of 0.6-0.9, and incubated for 15 min at 42 ^{o}C to induce the phage recombination RED genes whose expression is under control of a temperature-sensitive λ-repressor. Cells were cooled immediately by chilling on ice for 20 min, then centrifuged at 4000 rpm for 10 min and washed three times with sterile ice-cold water. Cells were resuspended in an appropriate volume of ice cold sterile water. Cells were either used fresh for electroporation or mixed with 30% sterile glycerol and stored at -80 ^{o}C for future use. The electroporation of 40 µl cells in a 0.2 cm gap electroporation cuvette was carried out in a Gene Pulser (BioRad) with 120 ng of REC DNA, using a pulse of 2.5 kV (at 25 µF and 200 Ω). Immediately after electroporation, 0.5 ml of SOC medium (2 % Bacto-tryptone, 0.5% , Bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose, pH 7.0) was added and cells were incubated at 32 ^{o}C for 1 h with gentle shaking (120 rpm) to initiate recombineering, recover and express antibiotic resistance. Cells were subsequently plated on LB medium containing 50 µg/ ml^{-1} kanamycin (Km) and incubated overnight at 30 ^{o}C. Transformants were tested for sensitivity to chloramphenicol; correct recombination of the REC vector into the BAC vector disrupts the CAT gene. Further verification of generated BIBAC vectors was carried out by restriction enzyme digests and PCR.

4.2.3. Fungal transformation

BIBAC constructs were introduced by standard electroporation into *A. tumefaciens* strain COR309 or COR308, and a fresh colony was grown overnight at 28 ^{o}C in LC medium (0.8% NaCl w/v, 1% Bacto-tryptone w/v, 0.5% Bacto-yeast extract w/v) supplemented with 5µg ml^{-1}

tetracycline (Tc) to select for the helper plasmid pCH32 and 50 µg Km ml^{-1} to maintain the BIBAC construct. Five ml of BIBAC-containing *A. tumefaciens* culture was spun down for 10 minutes at 4000 rpm and the pellet resuspended in 5 ml induction medium (IM, minimal medium as in Takken et al. (2004), supplemented with 40 mM MES, 0.5% glycerol, 0.2% glucose). After centrifugation as above, the pellet was resuspended to an OD600 of 0.4 in 5 ml IM containing the appropriate antibiotics and supplemented with 200 µM acetosyringone (AS, PhytoTechnology Laboratories, Shawnee Mission, KS). Cells were incubated at 28 ^{o}C for 6-8 hours to reach OD600 of 0.5. Control cells were treated identically in the same medium but without AS. *U. hordei* strain Uh364 and *U. maydis* strain 324, grown in 5 ml complete medium (CM; Holliday. 1974) for 2 days, were used to re-inoculate 20 ml fresh CM to an OD600 of 0.15 and subsequently grown to an OD600 of 0.5. *U. hordei* was always incubated at 22 ^{o}C and *U. maydis* at 28 ^{o}C unless mentioned otherwise. Both *U. hordei* and *U. maydis* cell cultures were diluted 10-fold in IM and mixed with an equal volume of AS-induced *A. tumefaciens* culture and 200 µl of the mixture was plated onto ME-25 filters (Schleicher and Schuell, 0.45 µm pore size, 47 mm diameter) which were placed on co-cultivation medium plates (IM but with 0.1% glucose and 200 µM AS added); negative controls contained no AS. The membranes were air-dried briefly for 10-60 minutes and incubated at 20-24 ^{o}C for 2-5 days on co-cultivation media. To select for transformants, membranes were transferred to CM plates containing Cefotaxime 200 µg ml^{-1} to kill off *A. tumefaciens* and 300 µg ml^{-1} hygromycin B (hyg B, Calbiochem EMD Biosciences, Inc. La Jolla, CA) to select for fungal transformants. After 4 days on selection media, individual transformants were transferred to CM medium supplemented with 300 µg ml^{-1} hyg B.

4.2.4. Analysis of transformants

Genomic DNA isolation and PCR amplification – Individual putative *U. hordei* and *U. maydis* transformants were grown in 5 ml CM medium supplemented with 300 µg hyg B ml^{-1} for two days. Subsequently, 1 ml of this culture was inoculated in 100 ml fresh CM medium with the same antibiotic. Cultures were spun down and pellets frozen rapidly in liquid nitrogen for direct use or storage at -80 ^{o}C. Frozen pellets were ground to a fine powder in liquid nitrogen and used for DNA extraction using the DNeasy Plant Maxi kit (QIAGEN) following the manufacturer's instructions. PCR was used to confirm the presence of T-DNA by amplifying an internal 1023 bp

fragment of the hygromycin B phosphotransferase open reading frame using primers hyg B-F (GTACCATGGAAAAGCCTGAACTCACCGCGACG) and hyg B-R (GCATCTAGACTCTATTCCTTTGCCCTCGGAC). The cycling conditions were as follows: an initial denaturation step of 5 min at 95 ^{o}C followed by 35 cycles of 30 sec at 95 ^{o}C, 30 sec at 60 ^{o}C and 1 min at 72 ^{o}C, and a final extension at 72 ^{o}C for 10 min. To verify the presence of an intact T-DNA insertion, another PCR reaction was performed using primers near the left border: cat-f2 (see above) and LB-r2 (CACAGCGACTTATTCACACGA). An intact left T-DNA border would result in the amplification of a 302 bp fragment. The cycling conditions were the same as above except for an annealing temperature of 64 ^{o}C and extension time of 30 sec.

DNA gel blot hybridization – For DNA blot analysis, 8 µg of genomic DNA was digested with AvaI or BglII run out slowly on a 0.8 % (w/v) agarose gel in 1X Tris-borate-EDTA (TBE) buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA pH 8.0), and transferred overnight to a Hybond-N+ membrane as recommended by the supplier (Amersham Biosciences/GE healthcare). Two hybridization probes were used: for the left border of the T-DNA, a fragment of 302 bp was amplified from BIBAC_2-1 by PCR using Taq polymerase and primers cat-f2 and LB-r2, and for right border of the T-DNA, a fragment of 564 bp was generated by PCR using primers cat-r2 (ACAAACGGCATGATGAACCT) and Rb-r2 (CACAGCGACTTATTCACACGA). These fragments were gel-purified using a QIAquick Gel extraction kit (QIAGEN) following the manufacturer's instruction. Probe labeling was carried out with αP^{32}-dCTP, using the Rediprime II Random Prime Labeling system (Amersham Biosciences/GE healthcare), and hybridized to the membrane using ULTRA-hyb buffer (Ambion) at 42 ^{o}C according to manufacturer's instructions. The blots were washed twice for 5 minutes with 2X SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0), 0.1% w/v sodium dodecyl sulphate (SDS) followed by two washes each of 15 min in 0.1 X SSC, 0.1% SDS. All washes were carried out at 42 ^{o}C and the blots were exposed to Kodak Biobax film (Kodak Canada, Toronto, ON, Canada).

4.3. Results

4.3.1. Recombineering

The recombineering technology is based on the RED homologous recombination system and uses functions that are provided by a defective λ prophage that is present on chromosomal DNA of *E. coli* strain SW102. These λ prophage gene products supply the functions that protect and integrate the linear introduced REC DNA into BAC vectors (Warming, et al. 2005). To convert BAC vectors into BIBACs, *Agrobacterium*-specific functions such as a bacterial selectable marker (kanamycin resistance), T-DNA specific border sequences BL and BR and a broad-host range origin of replication need to be introduced on the linear, recombining part of the REC vector. Flanking this transforming fragment are 40 bp ends providing the homologous termini for integration into the CAT resistance gene. Such REC vector was developed by Takken et al. (2004) for use in ascomycete fungi by introducing a fungal-specific selectable resistance cassette to allow AMT of *Fusarium* and *Aspergillus* species. I used the pFT41 backbone to convert a previously developed *Ustilago*-specific BAC vector, pUsBAC5, already containing an *Ustilago*-specific hygromycin B cassette under the control of the HSP70 promoter and terminator signals (Wang, et al. 1988). Integration of the REC fragment from pFT41 would create a binary construct that has the complete pUsBAC5 construct including the hygromycin B cassette and any genomic insert residing on pUsBAC5, in between the T-DNA borders BR and BL (Fig. 4.1).

Two different target constructs were made in *Ustilago*-specific BAC vector, pUsBAC5, by inserting a 11 kb Sac1 and a 9.3 kb Xba1 fragment from the *U. hordei* avirulence gene 1 (*UhAvr1*)-containing genomic region (Linning et al. 2004). The 11 kb SacI-fragment was cloned in the SacI site of pUsBAC5, creating pUsBAC5_1-6, and the 9.3 kb Xba1 fragment was inserted in the HindIII site by partially filling in 2 base pairs in each of the 5-overhanging tails generated by HindIII and XbaI with the Klenow fragment of DNA polymerase 1 to create only 2 bp-sticky overhangs (Korch 1987) ligation generated construct pUSBAC5_2-1. pUsBAC5 was used as an "empty vector" control. The introduction via electroporation of 120 ng of linear, PCR-amplified REC DNA into *E. coli* strain SW102 previously transformed with the BAC target constructs and heat-induced to activate the λ RED genes, usually resulted in 50-100 kanamycin-resistant colonies. No colonies were obtained from cells that were not heat-induced. Kanamycin resistance colonies were tested for chloramphenicol-sensitivity to select for proper recombination

in the CAT gene of the BAC vector. In general, 25 to 30% of kanamycin-resistant colonies became chloramphenicol-sensitive in RED induced cells (Table 4.1). Figure 4.2 shows an EcoRI restriction enzyme pattern of BIBAC plasmids purified from colonies that were chloramphenicol-sensitive and kanamycin-resistant. The restriction analysis of these recombinants showed that the REC vector had integrated at the proper position without causing any rearrangements of the BAC clones. These data demonstrate that recombineering works well and allows for the conversion of *U. hordei* BAC library clones into BIBAC vectors.

4.3.2. Fungal transformation

Constructs BIBAC_2-1, BIBAC_1-6 and BIBAC_5 ("empty vector" control), were introduced into *A. tumefaciens* strain COR309 and COR308 via electroporation. These *Agrobacterium* strains are *recA*$^-$ and contain extra copies of *virA* and *virG* on a helper plasmid. Frary and Hamilton (2001) have shown that extra copies of these AS inducer-sensing and signaling components are essential for successful transformation of plants with large pieces of DNA such as contained on BIBAC vectors. Co-cultivation of *Agrobacterium* cells harbouring BIBAC_5, BIBAC_2-1 and BIBAC_1-6 with *U. hordei* strain Uh364 in the presence of AS led to the formation of hygromycin B-resistant colonies, while no colonies were obtained on co-cultivation medium without AS. I also included the related corn smut pathogen *U. maydis* strain 324 (*a2b2*) for comparison. The AMT transfer efficiency of these BIBAC constructs into *U. hordei* is lower than that for *U. maydis* (Table 4.1). Interestingly, for *U. maydis*, some hygromycin B-resistant colonies were obtained in the absence of AS in co-cultivation medium, although AS was used in the induction medium. For *U. hordei*, hygromycin B-resistant colonies were obtained only when AS was used in both induction and co-cultivation media. I tested whether *U. hordei* cells without cell walls (protoplasts) would be more sensitive to the *Agrobacterium* T-DNA transfer machinery. AMT transformation efficiencies were found to be essential the same as for sporidia (Table 4.1). The transformation frequency of BIBAC_5, the "empty vector" control which still harboured a T-DNA insert of approximately 10 kb, was on average three times higher in *U. hordei* than that of BIBAC_2-1 and BIBAC_1-6 (Table 4.1). Various parameters have been reported to affect AMT efficiencies in other fungi, such as drying of co-cultivation medium plates with *Agrobacterium* and fungal mixtures for various times (Almeida, et al. 2007), the length of cocultivation period (Rho, et al. 2001), the use of different ratios of *Agrobacterium* to

fungal cells (Michielse et al. 2005b), and various cocultivation temperatures (Michielse et al. 2005b). However, due to low overall transformation efficiencies, no significant differences could be measured.

4.3.3. Molecular analysis of fungal transformants

To test the mitotic stability of the transgenes, eight randomly selected, hygromycin B-resistant transformants (selected on CM medium supplemented with 300 μg hygromycin B ml^{-1}) were transferred to selection-free PDA plates for 5 successive cycles (4 days of growth at 22 ^{o}C per cycle). Cells from non-selective PDA plates were then transferred to 100 ml CM liquid medium supplemented with 300 μg hygromycin B ml^{-1} for total genomic DNA isolation. To confirm the presence of intact T-DNA, two PCR analyses were performed: one to test for the presence of the internal hygromycin B phosphotransferase gene and one for the left T-DNA border. Using the primers hyg B-F and hyg B-R, a PCR product of expected size (1020 bp) was amplified from DNA of all eight putative transformants (Fig 4.3A) which correlated with the observed growth in selective hygromycin B medium. *Agrobacterium* generates T-DNA directionally from an initial nick at the right border which is then linked to the virD2 protein, ending at the left border. Integrated T-DNA therefore frequently has variable left border truncations in contrast to the right border junction which is often more precise (Tinland 1996; Bundock and Hooykaas 1996; Zhong et al. 2007). The second PCR was performed to verify the presence of intact left border sequences by using primers LB-r2 and cat-f2, which is expected to amplify 302 bp immediately adjacent to the left border of the T-DNA. Seven out of the eight transformants amplified a PCR product of the expected size (Fig 4.3B).

The genomic DNA of the eight selected, PCR-positive, stable *U. hordei* transformants was analyzed on DNA blots to determine the extent of random T-DNA integration events and assess copy number of the insertions. I analyzed both ends of the T-DNA insertion by hybridizing two separate blots with a left T-DNA border- or a right border-specific probe. Transformant C which was negative for the left border in the PCR analysis, was positive upon hybridization (Fig. 4.4B). Single fragments were revealed for all transformants for both right border and left border junctions, indicating that the T-DNA had inserted as a single copy in each strain, since the selected probes did not span the chosen restriction enzyme sites. At least seven of the transformants revealed junction fragments of different sizes indicating random insertion

events at different locations in the genome. One transformant, Uh364 BIBAC_1-6 transformant Z, did not show a positive hybridization signal with the right border-specific probe (Fig. 4.4C); in this case it is likely that the right border end of the T-DNA became truncated upon the integration event thereby deleting the probe binding site. Truncation of T-DNA upon integration is not uncommon but is normally more prominent at left border junctions.

4.4. Discussion

The main objective of this work was to establish an *Agrobacterium*-mediated transformation (AMT) protocol for transferring large fragments of genomic DNA, contained on BAC library clones, into the barley smut fungus, *Ustilago hordei*. To this end I evaluated the use of an *in vivo* recombineering method for converting BAC library clones into binary BAC (BIBAC) vectors and to subsequently develop an AMT protocol for this fungus. The recombineering method is based on a modification of cloned DNA in *E. coli* via a λ RED mediated homologous recombination and avoids the cumbersome restriction and ligation reactions usually carried out to modify DNA (Warming, et al. 2005). The recombination of the REC vector into a BAC clone is based on the expression of RED genes from a stably integrated defective λ prophage under the control of a temperature sensitive repressor, cI857. The REC vector provides all the required functions for the construction of a binary vector (Takken, et al. 2004). Recombineering uses stretches of homologous DNA which in this method is provided by the bacterial chloramphenicol resistance gene. Proper insertion of the REC vector into BAC clones results in the loss of chloramphenicol resistance while resistance to kanamycin, present on the REC vector, is gained. This provides an easy tool to select for likely proper recombinants which can then be verified by restriction enzyme analysis. I obtained an efficiency of proper recombination of 25-34% based on gain of kanamycin and loss of chloramphenicol resistance. This is comparable to the 40% reported for the conversion of *Fusarium oxysporum* and *Aspergillus awamori* BAC clones into BIBACs by recombineering (Takken, et al. 2004). The remaining colonies, found to be both kanamycin and chloramphenicol resistant, may be the result of integration of the transforming REC fragment into BAC vector locations other than the CAT gene, including homologous stretches in the BAC genomic insert, or may be due to dimerization of the generated BIBAC with the original BAC clone and thus contain both selectable markers (Takken, et al. 2004). Yu

et al. (2000) reported that such dimerization can be suppressed by transformation of both REC and BAC plasmids at the same time.

Compared to plant transformation, AMT of fungi is relatively new (Bundock, et al. 1995, de Groot, et al. 1998) but has been very successful for a number of species and the number of fungi that can be transferred by *Agrobacterium* is still increasing. However, optimization of the transformation protocol is required for each species (Amey, et al. 2002, Mata, et al. 2007, Michielse, et al. 2005a, Michielse, et al. 2005b). To our knowledge, this is the first report on AMT of the barley pathogen *U. hordei*.

U. hordei strains Uh364 and *U. maydis* strain 324 were transformed with either BIBAC_2-1, BIBAC_1-6 or "empty-vector" control BIBAC_5, using two *Agrobacterium* strains, COR308 and COR309. Overall, the transformation efficiency of *U. hordei* was low compared to *U. maydis* using the same protocol. In a recent study, Ji et al. (2010) reported on the development of an efficient AMT method for *U. maydis*, employing a series of optimization steps. In my study, no difference in transformation efficiencies was observed when using the two *Agrobacterium* strains COR309 or COR308, indicating that the origin of the virulence functions on the Ti plasmid do not influence efficiency. The transformation efficiency of BIBAC_5 was at least three times higher than that of both BIBAC_2-1 and BIBAC_1-6; it was slightly higher for BIBAC_2-1 compared to BIBAC_1-6. This suggests that the size of the insert (T-DNA) in the vector could influence the transformation efficiency. Alternatively, the inserts in both BIBAC_2-1 and BIBAC_1-6 clones originate from a region in the *U. hordei* genome where many repeats and transposable elements have been found and this could also be the reason for the low transformation efficiencies. Such elements might inhibit integration or, additionally, it may be that such repeats present on these T-DNAs and homologies in the genomic DNA in *U. hordei* make integration in the genome less efficient as to avoid duplication (Yu et al. 2000). Takken et al. (2004) also observed a low transformation efficiency and truncation of integrated T-DNA when using BAC clones containing genomic DNA inserts with large stretches of homologous DNA, compared with the empty vector without any homologous region.

I evaluated the effect of acetosyringone (AS), a plant phenolic compound that is produced in wound sites of plants. It is an inducer of the *vir* genes in *A. tumefaciens* (Gelvin 2003) and serves as inducing agent for *in vitro* transformation. I obtained *U. hordei* hygromycin B resistant colonies only when AS was used both in the induction and co-cultivation media, which suggest

that AS is essential for AMT of *U. hordei*, a requirement found for the majority of fungal species (Amey, et al. 2002, Michielse, et al. 2005a, Michielse, et al. 2005b, Duarte, et al. 2007, Marchand, et al. 2007, Zhang, et al. 2008). The presence of AS in induction medium before co-cultivation is not necessary for *Agrobacterium* growth but it has been reported to improve transformation efficiencies in *A. carbonarius*, *F. oxysporum* and *M. grisea* (Morioka, et al. 2006, Mullins, et al. 2001). Indeed, in parallel experiments of AMT transformation of *U. maydis* 324, I obtained a few hygromycin B resistant colonies in the absence of AS in the co-cultivation media, although AS was included in the induction medium.

The mitotic stability of the transformants was verified by growth on non-selective media plates for five successive transfers and subsequent comparative growth on selective versus non-selective medium plates. Subsequent PCR amplification of the hyg B phosphotransferase gene and the left T-DNA border and analysis of the genomic DNA by DNA blot hybridization was consistent with stable T-DNA integration into chromosomal DNA previously reported for T-DNA transfer to filamentous fungi (Gelvin 2003, Ji et al. 2010, Gelvin. 2003, Covert, et al. 2001). Previous reports showed that several parameters affect the T-DNA copy number in fungal genome (Michielse et al. 2005b). For example, the addition of AS in IM and the length of cocultivation time seem to affect the number of T-DNA integrations per transformant (Combier, et al. 2003, Rho, et al. 2001). The addition of AS in IM as carried out in our experiments, reduced the occurrence of multiple integrations in the ectomycorhizal fungus, *Hebeloma cylindrosporum* (Combier, et al. 2003), while in *M. grisea* the addition of AS in IM increased multiple integrations (Rho, et al. 2001).

DNA blot analysis revealed at least seven fragments of different sizes among the eight transformants analyzed, suggesting a random mode of integration. It has been suggested that the mode of T-DNA integration either by homologous or non-homologous recombination depends on the organism (Bundock, et al. 1995, Covert, et al. 2001, van Attikum, et al. 2001). Cloning and sequencing of the junctions between the integrated T-DNA and the genomic insertion sites would be necessary to verify true random integration and to assess the precise mode of integration.

The T-DNA transfer process in *Agrobacterium* starts at the right border after nicking and the attachment of a VirD2 protein on the 5′-end of the nascent single strand. The production of the T-DNA molecule proceeds until the left border sequence is reached and another nick is

introduced, generating an unprotected end (Tzfira and Citovsky 2006). After transfer and during integration, deletion of T-DNA nucleotides occurs at the junctions of the T-DNA repeats, most frequently at the unprotected left border end in plants, yeast and other filamentous fungi (Bundock and Hooykaas 1996, de Groot, et al. 1998, Tinland 1996, Zhong, et al. 2007). Recipient insertion sites in the genomic DNA also often suffer deletions. PCR analysis revealed that the left border end was missing from only one of the transformants but the DNA blot analysis showed that this must be a minor truncation of T-DNA at this left border (including a primer binding site) because the left border end probe still hybridized.

In conclusion, the strategy of using recombineering to convert BAC library clones into BIBAC constructs and to use *A. tumefaciens* for the transfer of these BIBAC constructs to *U. hordei* is feasible. We have several BAC libraries containing *U. hordei* genomic inserts of various sizes (Bakkeren et al. 2006). One is derived from a strain which has several avirulence genes and the complete genome of which has been sequenced (Laurie, J., Ali, S Linning, R., Bakkeren, G., Schirawski, J, Kahmann, R. *et al* in preparation). The method described in this paper will facilitate the functional analyses of individual genes and whole gene clusters by complementation studies.

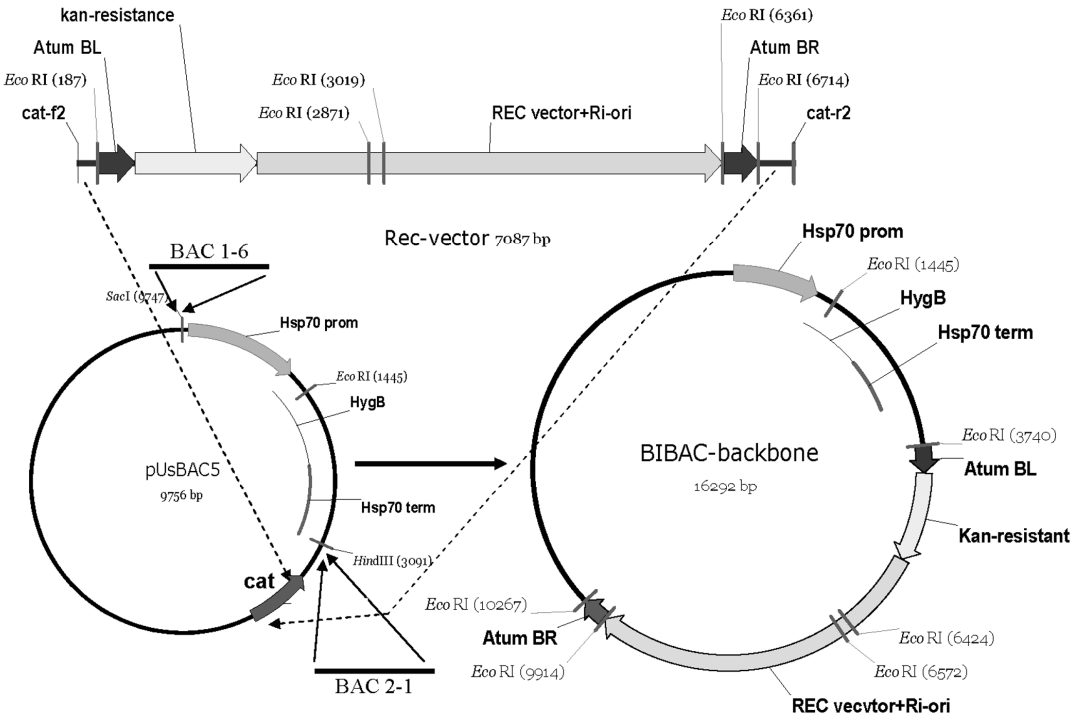


Figure 4.1 Schematic representation of the BAC to BIBAC conversion method. Conversion of Bacterial Artificial Chromosome vector, pUSBAC5, harbouring genomic inserts, into a binary BAC (BIBAC) vector using a linear 7,087 bp, PCR-amplified DNA fragment (REC-vector) from pFT41 (top line; Takken et al. 2004). The REC-vector recombines into the chloramphenicol resistance gene (CAT) present on pUSBAC5, using homologous regions present on both left (cat-f2) and right end (cat-r2) of the REC-vector (dashed arrows). Recombinants are selected for kanamycin resistance (Kan) present on the REC-vector and screened for loss of chloramphenicol resistance indicating proper integration. The location of the *U. hordei* genomic fragments in the respective BAC clones, 1-6 in the SacI site or 2-1 in the HindIII site, are indicated (solid arrows). The resulting BIBAC constructs are then transformed into a suitable *Agrobacterium* strain for subsequent transformation into *U. hordei*. Any DNA present between the right border (BR) and left border (BL) sequence elements is considered T-DNA and is transferred by *Agrobacterium* to the host. *U. hordei* transformants were selected on hygromycin B; a *Ustilago*-specific hyg B phosphotransferase-cassette present on the T-DNA resulted from recombination of the REC-vector with pUSBAC5.

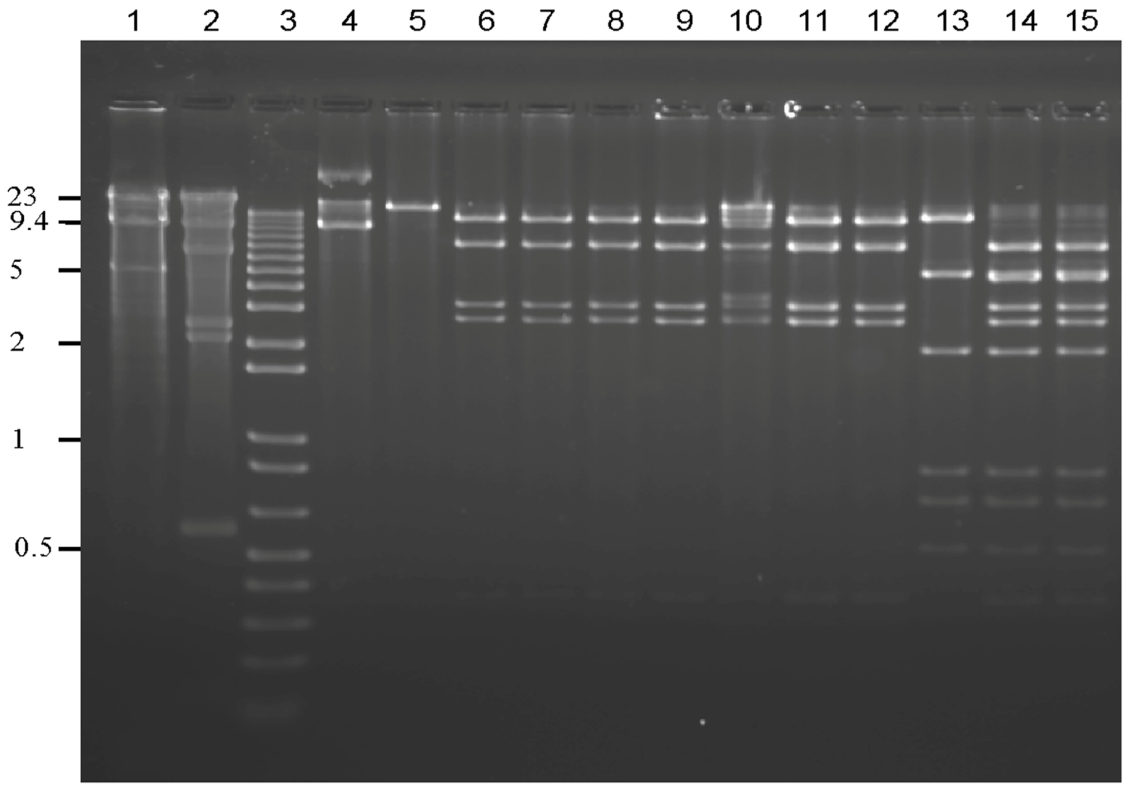


Figure 4.2 Verification of conversion of BAC clones to BIBAC vectors. Ethidium bromide-stained 1.2% agarose gel showing EcoRI-digested pUSBAC5 (lane 4), pUSBAC5_2-1 (lane 5), seven independent BIBAC_2-1 clones (lanes 6-12), pUSBAC5_1-6 (Lane 13), and two BIBAC_1-6 clones (lanes 14 and 15). The molecular markers are: 5 kb ladder (lane 1), λHindIII fragments (lane 2) and 1kb ladder (lane 3) with sizes indicated in kb on the left.

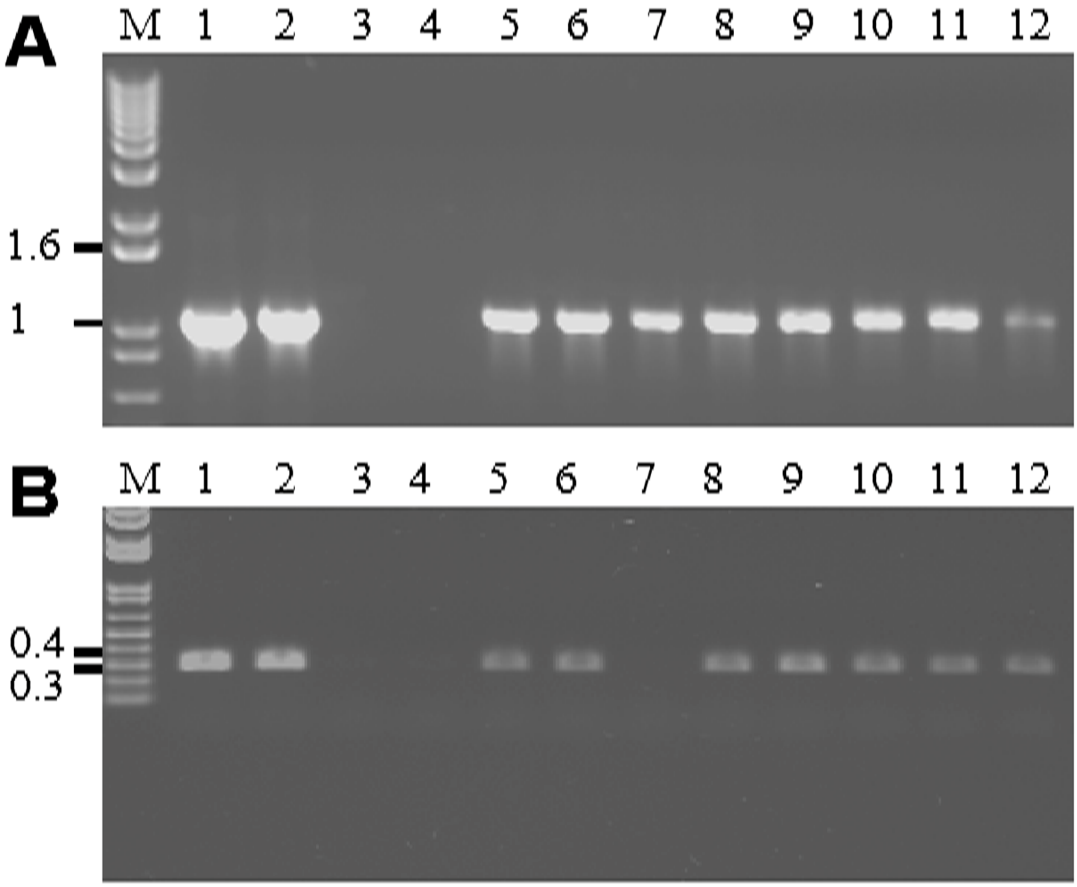


Figure 4.3 PCR analysis of genomic DNA of six independent BIBAC_2-1 and two independent BIBAC_1-6 *U. hordei* transformants. (A) Ethidium bromide-stained 1% agarose gel showing the amplification of a 1020 bp fragment constituting the hygromycin B phosphotransferase open reading frame, using primers hyg B-F and hyg B-R. (B) Ethidium bromide-stained 1% agarose gel showing PCR amplification products of 302 bp using primers LB-r2 and cat-f2, representing the T-DNA left border end. Lane 1: BIBAC_1-6 vector (positive control), lane 2: BIBAC_2-1 vector (positive control), lane 3: Uh364 untransformed (negative control), lane 4: Uh365 untransformed (negative control). Lanes 5-10: independent Uh364 BIBAC_2-1 transformants (named A, B, C, D, E and F, respectively), lanes 11 and 12: independent Uh364 BIBAC_1-6 transformants (named Y and Z, respectively). M: 1 kb plus molecular weight DNA ladder. Size bars at the left side of the gels are in kbp.

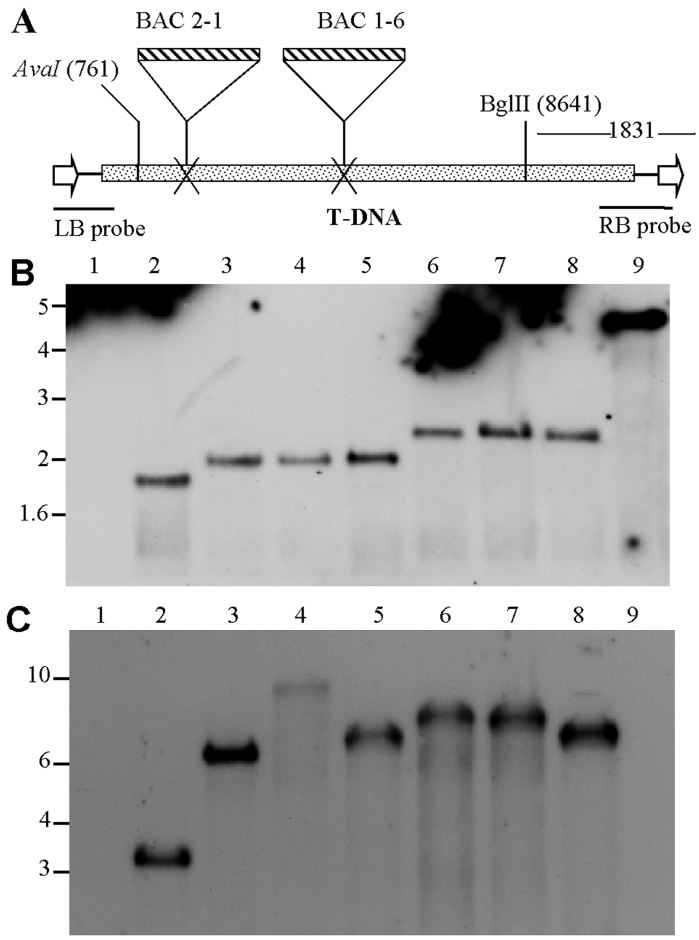


Figure 4.4 DNA blot analysis of the genomic DNA of independent *U. hordei* BIBAC transformants. (A) Genomic DNA was digested with AvaI, which cuts 761 bp proximal to the left border of the T-DNA. The membrane was probed with a 302 bp-fragment located within the left border of the T-DNA and the most-proximal AvaI site (black line in the cartoon above the blot; the location of the genomic inserts, BAC_2-1 or BAC_1-6, is indicated). (B) Genomic DNA was digested with BglII, which cuts 1831 bp proximal to the right border of the T-DNA. The membrane was probed with a 564 bp-fragment located within the right border of the T-DNA and the most-proximal BglII site (black line in the cartoon above the blot). Lane 1: Uh364 untransformed (negative control), lanes 2-7: Uh364 BIBAC_2-1 transformants A, B, C, D, E and F, respectively, lanes 8 and 9: Uh364 BIBAC_1-6 transformants Y and Z. Size bars at the left side of the blots are in kbp.

Table 4.1 Recombineering and transformation efficiencies

BAC	**RE (%)**	**Inoculation medium** [1]	**Cocultivation medium**	***U. hordei* TF (transformants/ 1.2x10^4 sporidia**	***U. hordei* TF (transformants/ 1.2x10^4 protoplasts**	***U. maydis* TF (transformants/ 1.2x10^4 sporidia**
2-1	30	+AS	-AS	0	0	0.4
		+AS	+AS	1	1	32
1-6	25	+AS	-AS	0	0	0.5
		+AS	+AS	0.8	0.9	27
pUSBAC5	34	+AS	+AS	3	NT	40

Recombination efficiencies of conversion of BAC clones into binary BAC constructs in *E. coli* (RE), and transformation efficiencies (TF) of *U. hordei* sporidia, protoplasts and of *U. maydis* sporidia using AMT. [1] AS, medium with (+) and without (-) acetosyringone added**.** NT, not tested.

CHAPTER 5

Towards the cloning of *UhAVR6*

5.1. Introduction

Avirulence (*Avr*) genes from different pathogens do not share sequence significantly with each others. Many do not have annotated homologs in public databases, although similar sequences can often be found in genomic sequences of related species. Consequently, identification of *Avr* genes is a constant challenge (Gan, et al. 2010b, Van't Slot and Knogge 2002). Several *Avr* genes have been isolated by classical genetics techniques from pathogens with small genomes such as bacteria. Such methodologies included the transformation of a genomic library from an avirulent to a virulent strain, followed by subsequent testing for an HR response on host plants (Collmer 1998, Van den Ackerveken and Bonas 1997). Due to large genome sizes and inefficient transformation methods in fungi and other eukaryotic pathogens, this method cannot be used efficiently (Lauge, et al. 1998). The most common methods for the cloning fungal avirulence genes are reverse genetic techniques and map-based cloning.

Several types of molecular markers have been developed for determining DNA sequence variation within and among species. These include amplified fragment length polymorphisms (AFLP), restriction fragment length polymorphisms (RFLP), simple sequence repeats (SSR), randomly amplified polymorphic DNA (RAPD) and single nucleotide polymorphism (SNP) markers. These methods are based on the detection of polymorphisms through the analysis of total genomic DNA among various isolates and or progeny of crosses. Each of these techniques has advantages and limitations, and the choice of fingerprinting technique depends on its application. In this study to clone the *U. hordei UhAvr6* gene, I used a PCR-based marker approach. The marker-based approach has been successful in the cloning of several fungal genes, including *U. hordei Avr1* (Linning et al 2004).

I used 115 SSR primer pairs designed from the *U. maydis* genome sequence and 55 SSR primers pairs from *U. hordei* BAC clone end-sequences to find a marker linked to *UhAvr6*. Additionally, I used sets of AFLP and RAPD primers in an alternate approach. All primers were tested on the genomic DNA of the avirulent parent (*Avr6*) and virulent parent (*avr6*), as well as

on four pools of combined progeny, two for each of genotype *Avr6* and *avr6,* respectively. I also constructed a new population segregating for this locus from two *U. hordei* strains collected from geographically distant areas. Several of these primer pairs amplified polymorphic bands, in the parents and pools but unfortunately no linked markers were confirmed after testing on individual progeny.

5.2. Material and methods

5.2.1. DNA manipulation

DNA preparations were carried out as described in Chapter 2 section 4.2.4. Bulked pools were made by mixing equal amounts of genomic DNA from four progeny giving a final concentration of 10 ng μl^{-1}.

5.2.2. SSR analysis

115 SSR primer pairs (5 per chromosome) representing microsatellite markers of *U. maydis,* a closely related corn smut, were obtained from our collaborators (Munkacsi and May University of Minnesota). For the sequences of the primers see (Munkacsi, et al. 2008). 55 SSR primer pairs were designed for *U. hordei* based on sequences obtained from end-sequences of 2300 BAC clones from the avirulent parent Uh364 (*Avr6*) and are listed in Table 5.1. Primers were designed to all microsatellite repeat sequences identified and those pairs giving a 100-400 bp PCR product were retained. More than half of these primers were selected for trinucleotide repeats while the rest represent di-, tetra- and penta-nucleotide repeats. PCR reactions were carried out in 25 µl volumes containing 2 mM MgCl2, 100 µM of each of the four dNTPs, 0.5 unit of recombinant Taq polymerase (Invitrogen), 25 ng of genomic DNA as template in 1X PCR reaction buffer (Invitrogen) with 0.4 µM of each forward and reverse primer. The cycling conditions were as follows: an initial denaturation step of 5 min at 95 ^{o}C followed by 35 cycles of 30 sec at 95 ^{o}C, 30 sec at 55 ^{o}C and 1 min at 72 ^{o}C, and a final extension at 72 ^{o}C for 10 min. Reaction products were run on 2-4% agarose gels (MetaPhor) depending the size of the product, for 6-8 hours at 110-160 W. The running buffer consisted of 1 X TBE (45 mM Tris-Borate, 1mM EDTA, pH 8.0).

5.2.3. RAPD analysis

The set of RAPD primers was designed and synthesized at the Nucleic Acid and Protein Synthesis unit at UBC Vancouver. For RAPD analysis (Williams, et al. 1990), PCR reactions were carried out as described for SSR analysis (section 5.2.2), but using instead a single primer at a concentration of 0.6 μM. The reaction was carried out with an initial denaturation step of 5 min at 95 ^{o}C followed by 40 cycles of 12 sec at 95 ^{o}C, 60 sec at 36 ^{o}C, 60-sec ramp to 72 ^{o}C and 1 min at 72 ^{o}C, and a final extension at 72 ^{o}C for 10 min. The low annealing temperature was used for shorter primers (series 1-800) while a higher annealing temperature of 42 ^{o}C was used for longer primers (series 801-890). PCR products were run on 1.5% agrose gels in 1 X TBE.

5.2.4. AFLP analysis

AFLP analysis was carried out as described Linning, et al. (2004) and Vos, et al. (1995). In short, genomic DNA was digested by a combination of a "six-cutter" restriction enzyme, BamH1, and a "four-cutter" restriction enzyme, Mse1, followed by ligation with the corresponding adapters for these enzymes. The digested DNA was pre-amplified with three different primer combinations with one specific nucleotide (i) BamPc-MsePt, (ii) BamPc-MsePc and (iii) BamPt-MsePc. Twenty different primer combinations were subsequently used for each of these pre-amplified fragments having two specifying nucleotides at each primer end. The "six cutter" primers were labeled with [γ^{33}P] dATP (6000 Ci/ mMol, Perkin Elmer, LAS Canada Inc. Wood Bridge, Ontario, Canada) using the standard T4 polynucleotide kinase labeling procedure (Vos et al 1995). 4 μl of AFLP products were mixed 1:1 (v/v) with formamide and dye, heated at 95 ^{o}C for 5 min and then separated on a 4.5% polyacrylamide gel [the ratio of acrylamide and bis-acrylamide was 20:1 (w/w)], 7.5 M urea, 1X TBE at constant power of 110 W at 50 V/cm. Gels were dried and exposed to Kodak X-OMAT AR X-ray film.

5.3. Results

5.3.1. Construction of populations segregating for *UhAvr6* and *uhavr6*

Two populations were used in this study. One had been previously constructed to analyze the segregation of three avirulence genes (*UhAvr1*, *UhAvr2* and *UhAvr6*; Linning, et al. 2004). The

other one was constructed in this study, because it was revealed during the course of my screening experiments that the existing population was insufficiently genetic diverse with respect to finding polymorphisms as they related to the *UhAvr6* and *uhavr6* alleles. This new population was generated by crossing two haploid strains: Uh365 (isolated from southern Manitoba, Canada) and Uh813 (isolated from Iran) on universal susceptible barley cultivar Odessa. Prior to the final selection of the parents, I screened six geographically diverse *U. hordei* strains using several RAPD and SSR primers to select ones that had increased genetic diversity. Teliospores were collected from infected plants; individual basidiospores were isolated and tested in mating tests against haploid basidiospores of known mating type in order to determine their mating type. Fifty-two progeny; 26 for mating type 1 and 26 for mating type 2, were selected for evaluation of their genotypes on barley cultivars Odessa (*ruh6*) and Plush (*Ruh6*) by backcrossing with the virulent partner of opposite mating type. Progeny producing infection of more than 10% on Odessa with no disease on Plush were considered to have the *UhAvr6* genotype. *U. hordei* strains having the *UhAvr6* allele do not produce disease on Plush, while the virulent strain (*uhavr6*) can produces up to 100% infection in control experiments (Linning, et al. 2004). Progeny producing more than 10% disease on both Odessa and Plush were categorized as virulent, having the *uhavr6* genotype.

5.3.2. Pools for bulked segregant analysis (BSA)

To identify markers linked to the *UhAvr6* or *uhavr6* alleles, a bulked segregant analysis or BSA (Michelmore, et al. 1991) approach was used. Four pools were made, two for each *UhAvr6* and *uhavr6* and each consisting of four progeny (Table 5.2). Small pools containing four progeny each were made in this study, in the hope that this would reveal weak linkage to *UhAvr6* which would indicate a possible location on the available *U. hordei* genome sequence. Only those progeny revealing a clear virulence or avirulence phenotype were selected for the pools, which is critical for the identification of linkage to the avirulence gene. Pools were made in such a way that the bulked progeny were uniform for mating type as well as the other avirulence genes, if known, such as *UhAvr1* and *UhAvr2*. In this manner, the pools differed only for the desired gene.

5.3.3. SSR primer screening

115 SSR primers pairs from *U. maydis* were used in PCR reactions on two parents and four pools with bulked genomic DNA from *UhAvr6* and *uhavr6* to screen for differences in amplification profiles. 56 (50%) of these primers produced fragments in *U. hordei*, showing that the two organisms are at least moderately related at the DNA level. These primers amplified from one to as many as five fragments with an average of three fragments per primer. Some of these primers also produced more than one fragment in *U. maydis* (Munkacsi, et al. 2008, Munkacsi AB University of Minnesota Thesis, 2005). The PCR fragments amplified by these primers were of different intensities; some were very strong while others were hardly visible in ethidium bromide-stained gels. The weak bands were more polymorphic, but not reproducible in different PCR reactions and could have resulted from mismatches at primer binding sites. Primers not producing products were re-tested to confirm that a lack of amplification was not a PCR reaction artifact. Consistent failure of primers may be attributed to the absence of a complementary binding site or prohibitive length of intervening sequences between primer pairs. Of the 56 successful primer pairs, ten gave a polymorphic band in both the parents and the pools (Table 5.3). In the next step, I screened all the available progeny of these populations for polymorphisms, but was unable to find any linked to *UhAvr6* or *uhavr6*. An example of a polymorphic banding pattern is shown in Figure 5.1.

In addition to the *U. maydis* SSR primers, I also used 55 SSR primer pairs designed from BAC end-sequences of *U. hordei* for PCR analysis of bulked genomic DNAs to investigate the difference in amplification profiles. All but one of the tested primer pairs (Table 5.3) produced fragments of expected sizes in the PCR reactions. Unlike the SSR primers from *U. maydis*, these primers produced a single product except for a few pairs which yielded two. Most of the PCR products were of uniform intensity in both the parents and pools. Only three of the primer pairs amplified polymorphic fragments in the parents and pools but none were found linked to *UhAvr6* or *uhavr6* after screening the individual progeny (Table 5.3, Fig. 5.1).

5.3.4. AFLP primer screening

The screening of SSR primers from *U. maydis* and *U. hordei* was unsuccessful in finding markers linked to *UhAvr6* or *uhavr6*. I decided to attempt the AFLP marker-based approach for the cloning of the *UhAvr6* gene in the population segregating for these avirulence genes. To find a marker linked to *UhAvr6* or *uhavr6*, I used 60 different primer combinations after digesting the bulked DNA with different restriction enzymes (see Materials and Methods). All the AFLP primers gave an average of 20 fragments per primer pair tested. Two of the tested pairs revealed a polymorphic fragment between the parents and pools (Fig. 5.1, Table 5.3). These primers were further analyzed on individual progeny for linkage to *UhAvr6* or *uhavr6* but none was seen (Table 5.3).

5.3.5. RAPD primer screening

The *UhAvr6* and *uhavr6* parents and progeny pools were screened for polymorphisms with more than 200 RAPD primers. 150 of these primers yielded several fragments ranging from 1 to 13 with an average of six fragments per primer. To detect polymorphisms, I looked carefully to find differences in the sizes of fragments and/or the presence/absence of fragments in corresponding genotype pools. These amplified products varied in length from 150 to 3500 bp. The intensities of the bands were also not uniforme; some bands were very bright while others were very faint in the ethidium bromide-stained gels. The faint bands were also not reproducible while the stronger bands were more consistent across different PCR reactions. Fifty primers did not amplify any RAPD fragments during either the initial or confirmatory PCR reactions. I postulate that this could be due to the absence of complementary binding sites for these primers. Alternately, the primer binding sites could have been too widely separated and could not amplify the DNA in between by PCR under the conditions tested. In the initial analysis, several primers produced polymorphic fragments but most of them were faint and were not reproducible under different PCR reactions. Only two of the tested primers, 714 and 719, produced a polymorphic band in the pools, but after testing on individual progeny only 714 gave a polymorphic band that appeared to be weakly linked. The band that was present in the *UhAvr6* parent was excised from the gel, cloned and sequenced in order to design sequence-characterized amplified region (SCAR) primers for more robust visualization of the polymorphism; however, analysis with the SCAR

primers on individual progeny could not confirm the linkage, so this experimental approach was not pursued further.

5.4. Discussion

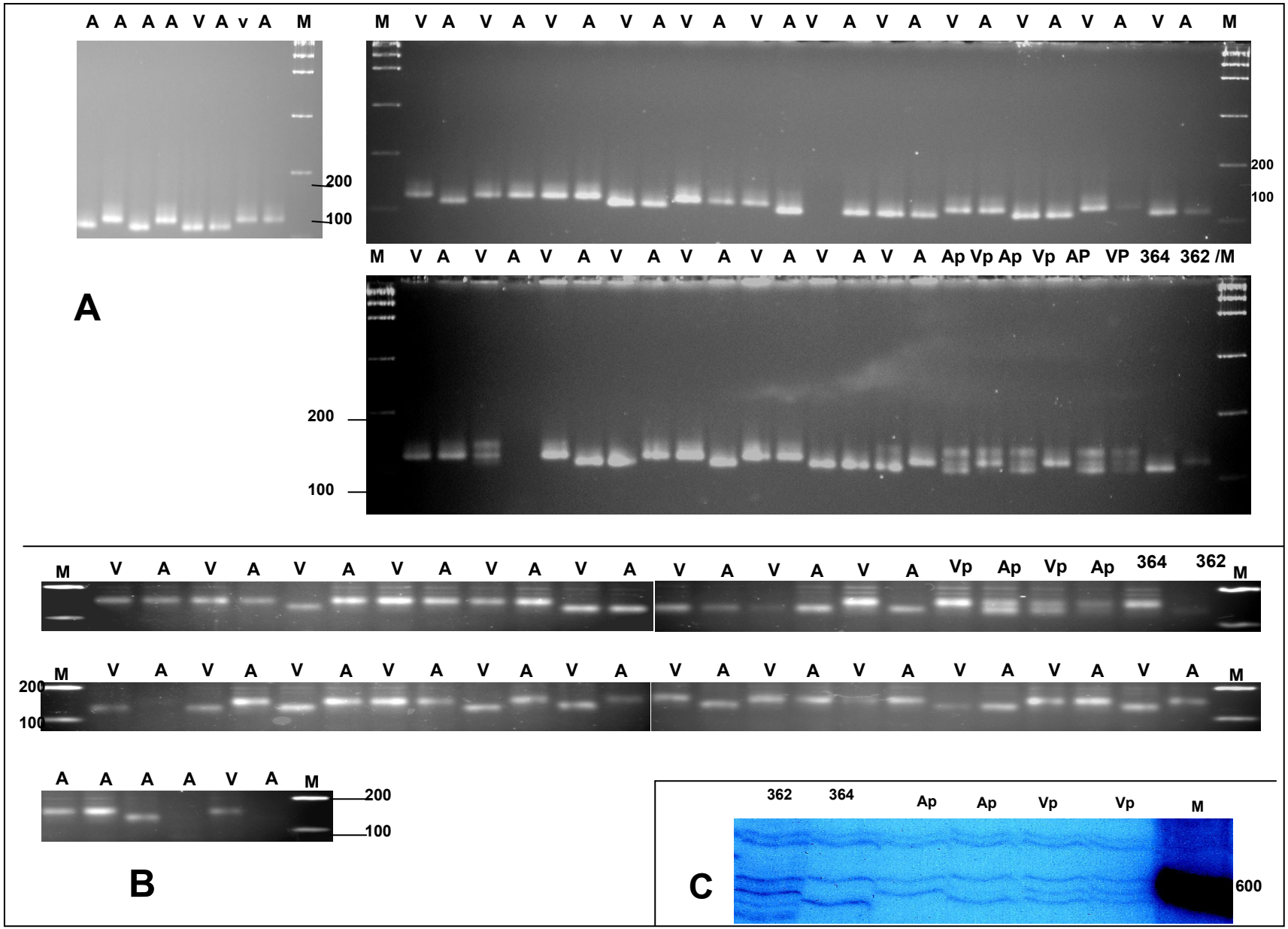
The existing population segregating for the dominant avirulence gene, *UhAvr6* did not yield a linked marker after testing many AFLP, RAPD and SSR markers. It became apparent that this population was not sufficiently genetically diverse as the two parents used to generate the population were back-crossed several times. In this study, a new population of *U. hordei* was generated which was segregating for the avirulence gene *UhAvr6*. In this new population, the *UhAvr6* and *uhavr6* alleles segregated independently from the mating-type locus, *MAT*, as has been shown in the previous populations (Linning, et al. 2004). Pathogenecity tests of back-crosses on Plush confirmed that the avirulence gene *UhAvr6* segregated as a dominant gene in the population.

BSA was used in this study to find markers linked to the *UhAvr6* gene in both populations segregating for this gene. The main principle behind BSA is the randomization of the genetic background of unlinked loci and the saturation of the region of interest (Michelmore, et al. 1991). The use of two pools for the same trait as used in this study, has been suggested because of the poor reproducibility of RAPD markers in some cases and to ensure that polymorphisms are not artifactual PCR variations (Ellsworth, et al. 1993, Penner, et al. 1993). Small pools of four progeny were made in this study because we hypothesized that by using smaller pool sizes, a weaker linkage could be identified, since a previous study found no markers linked to *UhAvr6* in pools of eight progeny (Linning, et al. 2004). However, it is acknowledged that in small pools the frequency of false positives is increased relative to that found for larger pools (Michelmore, et al. 1991). This may be the reason that we identified several polymorphic bands in the pools and progeny, but could not confirm them as linked to *UhAvr6* or *uhavr6* after testing them on individual progeny.

U. hordei SSR primers yielded fewer polymorphic fragments when compared to the *U. maydis* SSR primers. The reason for this may be that *U. hordei* SSR primers amplified comparatively fewer loci. These 54 primer pairs amplified a total of 65 loci while the 56 primer pairs from *U. maydis* amplified a total of 170 loci, almost three times as much. Generally, the

AFLP markers appeared to be more polymorphic when compared to the products obtained with the SSR primers**.** This could be because AFLP markers amplify many loci per reaction and can therefore quickly scan the whole genome, while SSR primers amplify fewer loci, necessitating a more comprehensive commitment to screen a genome. On the other hand, SSRs detect variations within repetitive DNA regions which are highly variable compared to the rest of the genome because of the slippage that can occur during recombination. This is more frequent than other types of mutations such as point mutations or insertion/deletions (Palombi and Damiano 2002).

From this study it is clear that the different virulent and avirulent strains of *U. hordei* are rather similar to one another at the *UhAvr6* locus and that a very small variation could be responsible for the avirulence/virulence allelic differences. In several pathogens, it has been shown that single base pair mutations are responsible for changing avirulence to a virulence phenotype (Joosten, et al. 1994, Parlange, et al. 2009, Schurch, et al. 2004, van Esse, et al. 2007, Westerink, et al. 2004). The sequence analysis of several predicted secreted proteins from different field isolates actually confirms this high degree of sequence homology between geographically diverse strains (Chapter 2). The molecular marker-based approaches that were used for the cloning of *UhAvr6* genes in this study, may not be very efficient for revealing point mutations. A possible alternative approach may be using molecular biological techniques that are efficient for revealing point mutations. One such technique is the Targeting Induced Local Lesions IN Genomes (TILLING) to reveal point mutations (Comai, et al. 2004, Till, et al. 2003a, Till, et al. 2003b). An alternative approach could be to sequence the genomes of different strains by next generation sequencing technologies which has become relatively cheap, and then search for single nucleotide polymorphisms (SNPs) and link them in the population.


A A A A V A v A M
200
100
M V A V A V A V A V A V A V A V A V A V A V A V A M
200
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M V A V A V A V A V A V A V A V A Ap Vp Ap Vp AP VP 364 362 /M
200
100
A
M V A V A V A V A V A V A V A V A V A Vp Ap Vp Ap 364 362 M
M V A V A V A V A V A V A V A V A V A V A V A V A V A M
200
100
A A A A V A M
200
100
B
362 364 Ap Ap Vp Vp M
600
C

Figure 5.1 PCR amplification with SSR and AFLP primers showing polymorphisms in parents, pools and progeny. (A) Ethidium bromide-stained agarose gel revealing polymorphisms generated by PCR amplification with an SSR primer pair from the *U. maydis* genome sequence as an example, on pooled progeny having dominant allele *UhAvr6* (AP), pooled progeny having recessive allele *uhavr6* (Vp), individual progeny having dominant allele *UhAvr6* (A), individual progeny having recessive allele *uhavr6* (V),, Parents were Uh362 (*uhavr6*) and Uh364 (*UhAvr6*). M; Marker (Low DNA mass ladder). (B) Ethidium-bromide-stained agrose gel revealing polymorphisms generated by PCR amplification with an SSR primer pair from the *U. hordei* BAC end sequences. (C) Section of an autoradiograph showing polymorphic AFLP markers in four BSA pools and parents. Size bars at the side of the blots are in bp.

Table 5.1 SSR and AFLP primers for *U. hordei* used in this study.

#	Primer Name	Clone Name	Contig No	Repeat type	Primer sequence	Product Size (bp)
829	Uh SSR IL	H001D03_CR	96	Trinucleotide	TCCTTTCAGAGCTTGCTAAC	224
830	Uh SSR IR				ACAGTACCACAGGTATTCGG	
831	Uh SSR 2L	H001G04_C7	1	Trinucleotide	ACTAGCATTCGCAATCTCAT	241
832	Uh SSR 2R				AGAAGAACGTGGCTATTGAG	
833	Uh SSR 3L	H001J18_C7	101	Trinucleotide	AGCTTTCAGAGCAGAGACAG	228
834	Uh SSR 3R				AGTATCCTCAACTGACAGCG	
835	Uh SSR 4L	H001M23_CR	37	Trinucleotide	TTTCCTATCGTCAACATCCT	389
836	Uh SSR 4R				ATCTTGCTTGAACAATGGAC	
837	Uh SSR 5L	H002B09_C7	100	Trinucleotide	GATTGGAGCAGTAGACAAGC	349
838	Uh SSR 5R				TGTCTTTGCCTCAACTACCT	
839	Uh SSR 6L	H002J09_CR	6	pentanucleotide	TTGGTAGTTCGGAGTAAGGA	264
840	Uh SSR 6R				TCACGTGCTGTACCTAGTTG	
841	Uh SSR 7L	H003M04_CR	not known	Trinucleotide	AGCTTTCAGAGCAGAGACAG	228
842	Uh SSR 7R				AGTATCCTCAACTGACAGCG	
843	Uh SSR 8L	H004F11_C7	3	Dinucleotide	GAATGAGGTCAAGAGTCAGC	137
844	Uh SSR 8R				CTCTTGGTGTCTTCTTGGAG	
845	Uh SSR 9L	H004F24_CR	62	Dinucleotide	cGACTGTGGTTGTGTATCTG	214
846	Uh SSR 9R				TCGTTGTTAGGTGGAGAGAT	
847	Uh SSR 10L	H004G03_CR	5	tetranucleotide	CTTGGCAAGGCTAATACCTA	373
848	Uh SSR 10R				ACTACCtttgGATTGCAAGA	
849	Uh SSR 11L	H004L07_C7	65	Dinucleotide	GGTCACTCGAGTAAGTCTGC	244
850	Uh SSR 11R				GACTGTCCTCGTCAACTTGT	
851	Uh SSR 12L	H006M20_C7	1	Trinucleotide	GCTGCTAGTCTTCCACACTT	353
852	Uh SSR 12R				ATATGGTTCCATCGTTTCAG	
853	Uh SSR 13L	H006N02_CR	9	pentanucleotide	GTTGAACGACCTCTCGTAAG	336
854	Uh SSR 13R				CCATCTTTGTCAGTCTGGAT	
855	Uh SSR 14L	H006P13_C7	10	Trinucleotide	GTAACCAACTTTGTCGGAAG	246
856	Uh SSR 14R				AAAGAATCATATCTCGCCAA	
857	Uh SSR 15L	H001A10_CR	57	Trinucleotide	ACATTTGGAGTCTGATGAGG	238
858	Uh SSR 15R				GATGTCGGAAGAGTTGTAGC	

#	Primer Name	Clone Name	Contig No	Repeat type	Primer sequence	Product Size (bp)
859	Uh SSR 16L	H001A13_CR	44	tetranucleotide	GTTAGACCTGCAATTGCTTT	304
860	Uh SSR 16R				CTTTGATCTGATGGGTGTCT	
861	Uh SSR 17L	H001A14_C7	20	Dinucleotide	GAGCGTAAGCAAGACCTAAA	304
862	Uh SSR 17R				GAAGCAAGGAGTTGAAGATG	
863	Uh SSR 18L	H001A21_C7	2	Trinucleotide	ATTGCTCCAGTAGGTCcc	253
864	Uh SSR 18R				CGTTCAGCTGTAATACCTCC	
865	Uh SSR 19L	H001B06_CR	23	Trinucleotide	TCACATCAACACTGACATCC	125
866	Uh SSR 19R				AGACAGCTTTCTCTAGGGCT	
867	Uh SSR 20L	H001C15_CR	95	Trinucleotide	AGAGCATCGTCAGATAGCAT	398
868	Uh SSR 20R				CAACGATGACAGTACAGAGC	
869	Uh SSR 21L	H001D12_C7	3	Dinucleotide	GTTCAAATTCGAGTCTCTGC	133
870	Uh SSR 21R				AAGAACGGCAAAGTCATAAA	
871	Uh SSR 22L	H001F03_CR	266	pentanucleotide	AAGATCAATGTACCACAGCC	111
872	Uh SSR 22R				CCAATCAATATGTATGTgcg	
873	Uh SSR 23L	H001G10_CR	5	pentanucleotide	TTACTGAGAGGCTCATTCGT	134
874	Uh SSR 23R				CAGGACCTTTCTGTATCTGC	
875	Uh SSR 24L	H001I05_CR	4	Dinucleotide	TGGATAAGGATCCACTTGAC	129
876	Uh SSR 24R				CCCTGTACAGTaCTCGATGAA	
877	Uh SSR 25L	H001L06_CR	101	Dinucleotide	CTGCAGCACAGTAGTCGTAA	125
878	Uh SSR 25R				GAAGCGATACTTCTTGGCTA	
879	Uh SSR 26L	H001M01_C7	76	Trinucleotide	GCTAGAATTTAGGTTGGTGC	108
880	Uh SSR 26R				CGAGCATGGTCTATTAGCTC	
881	Uh SSR 27L	H002C04_C7	8	Trinucleotide	GACCTCTGTGGACACTCTGT	135
882	Uh SSR 27R				ACATTCACCAGCCTTATCAC	
883	Uh SSR 28L	H002H17_CR	47	tetranucleotide	CTCGAAGAGGATGTAGTTGG	119
884	Uh SSR 28R				AAGGATTCAGGAAAGGAGAC	
885	Uh SSR 29L	H002K02_C7	100	Trinucleotide	AGGTTATGATGACACCAAGG	113
886	Uh SSR 29R				AAGCATCAAGTCAACACACA	
887	Uh SSR 30L	H002K10_C7	12	Dinucleotide	TGAAACTCTCCTCTTCCAAA	106
888	Uh SSR 30R				CAGAGGACAACAAAGAGGAG	
889	Uh SSR 31L	H002N01_CR	4	Trinucleotide	GAATGGAGTGGTGTTGCTAT	121

#	Primer Name	Clone Name	Contig No	Repeat type	Primer sequence	Product Size (bp)
890	Uh SSR 31R				CTGTGTAATTGATATTGACATTGA	
891	Uh SSR 32L	H002N17_C7	55	tetranucleotide	AGACGATGCTTTAGGGAt	104
892	Uh SSR 32R				GCGTCAACGAGAACGCAT	
893	Uh SSR 33L	H002O08_CR	86	pentanucleotide	TCACTGCTGTTGTTGTCATT	144
894	Uh SSR 33R				GCACAccTACAAAGAGAAGG	
895	Uh SSR 34L	H003H10_C7	115	Trinucleotide	GTTGGTGGGACTAGTTGAGA	142
896	Uh SSR 34R				GAACGAGGATGATGAACTGT	
897	Uh SSR 35L	H004A16_CR	20	pentanucleotide	TATGGATAAGGAGCAAAGGA	130
898	Uh SSR 35R				CTTGCTAACCTatCAGACGC	
899	Uh SSR 36L	H004A22_CR	9	Trinucleotide	TCACCATGTTTGGTTCTT	151
900	Uh SSR 36R				CTTACCTCCAGTCGTACCAA	
901	Uh SSR 37L	H004C11_CR	125	Trinucleotide	TCACATCAACACTGACATCC	125
902	Uh SSR 37R				AGACAGCTTTCTCTAGGGCT	
903	Uh SSR 38L	H004D14_C7	57	Dinucleotide	ACTTTACACGACCACGACTT	135
904	Uh SSR 38R				ACGTGATTACCATTCTCGAC	
905	Uh SSR 39L	H004H18_C7	12	Dinucleotide	TGAAACTCTCCTCTTCCAAA	106
906	Uh SSR 39R				CAGAGGACAACAAAGAGGAG	
907	Uh SSR 40L	H004I22_C7	10	Trinucleotide	TGAACGAAAGAGTGTGAGTG	118
908	Uh SSR 40R				GTCTTTCCTCCTCTCTCCAT	
909	Uh SSR 41L	H004K03_C7	44	Trinucleotide	AACCTCAATCTCAACCACAG	100
910	Uh SSR 41R				AGTAACGGCTGCTGATATTT	
911	Uh SSR 42L	H005A12_C7	2	Trinucleotide	ATTGTGTTGCAGTGGTGTTA	189
912	Uh SSR 42R				TGACTGATGACAAGACGGTA	
913	Uh SSR 43L	H005A14_C7	86	Trinucleotide	ATGTTGCCCAGATACAGAAG	199
914	Uh SSR 43R				TTTCCTTCTCCTCAACTGAA	
915	Uh SSR 44L	H005B12_CR	76	tetranucleotide	AAGGATTCAGGAAAGGAGAC	119
916	Uh SSR 44R				CTCGAAGAGGATGTAGTTGG	
917	Uh SSR 45L	H005C16_C7	55	Trinucleotide	ACTTTGCCTTTATCACTGGA	137
918	Uh SSR 45R				AAGGCACAACAACAGCTC	
919	Uh SSR 46L	H005D07_C7	96	pentanucleotide	CTATTGATGAAGAAGCCCAG	146
920	Uh SSR 46R				TCACTGAGATGTGAGGTTGA	

#	Primer Name	Clone Name	Contig No	Repeat type	Primer sequence	Product Size (bp)
921	Uh SSR 47L	H005I08_CR	47	Trinucleotide	ATGGTCGTCACGAGAATAAC	193
922	Uh SSR 47R				TCGTCGTAGAGACCAATACC	
923	Uh SSR 48L	H005I09_C7	55	tetranucleotide	GATGCTTTAGGGATGCag	100
575	Uh SSR 48R				GCGTCAACGAGAACGCAT	
576	Uh SSR 49L	H005K09_CR	86	Trinucleotide	TCACTGCTGTTGTTGTCATT	144
577	Uh SSR 49R				GCACACCTACAAAGAGAAGG	
578	Uh SSR 50L	H006K19_CR	6	Dinucleotide	CTCAAGGACGAAGTAACCAG	133
579	Uh SSR 50R				TTCGGATCACGTAACCTAAC	
580	Uh SSR 51L	H006L11_C7	65	Trinucleotide	CCAAAGGAACTGTCACTGAT	112
581	Uh SSR 51R				CTGTGcctcTTGAgctGT	
582	Uh SSR 52L	H001E02_CR	43	Trinucleotide	CATTCTGCGTATTGTTGATG	182
583	Uh SSR 52R				CATGTCTCCTTCTCTCTTCG	
584	Uh SSR 53L	H002P11_C7	43	Dinucleotide	AAGTGTTGTCCCGATAACTG	180
575	Uh SSR 53R				ATGGTCATTAAGTGGAATGC	
829	Uh SSR 54L	H002H22_CR	118	Trinucleotide	AGGAGAAAGAGCATGATGAA	205
830	Uh SSR 54R				CAGAAATGACTTTGCATTGA	
831	Uh SSR 55L	H003H10_C7	115	Dinucleotide	GACAGTTAGTGTGTCAGCGA	313
832	Uh SSR 55R				same primer like UhSSR34 R	
833	MsePt				GATGAGTCCTGAGTAAt	
834	BamPc				GGACTGCGTACGATCCc	
835	BamPt				GGACTGCGTACGATCCt	
836	BamPca				GGACTGCGTACGATCCca	
837	BamPcc				GGACTGCGTACGATCCcc	
838	BamPta				GGACTGCGTACGATCCta	
839	Adapter MseI MseAI				GACGATGAGTCCTGAG	
	Adapter MseI MseA2				TACTCAGGACTCAT	
	Adapter BamHI/Bgl II BamAI				CTCGTGGACTGCGTAC	
	Adapter BamHI/Bgl II BamA2				GATCGTACGCAGTCCAC	
	MsePt				GATGAGTCCTGAGTAAt	

#Primer number refer to Bakkeren Lab primer inventory

Table 5.2 Composition of pools used for bulked segregant analysis.

Pools from new population		
Pools or parent	Strain number with mating type I	Strain number with the mating type II
V6-1	852 (V6), 863 (V6)	848 (V6), 855 (V6)
V6-2	895 (V6), 912 (V6)	873 (V6), 898 (V6)
v6-3	878 (v6), 902 (v6)	861 (v6), 891 (v6)
v6-4	917 (v6), 924 (v6)	893 (v6), 897 (v6)
Parent		365 (V6)
Parent	813 (v6)	
Pools from old population		
V6-1	420 (V6), 398 (V6)	392 (V6), 407 (V6)
V6-2	411(V6), 427(V6)	385 (V6), 382 (V6)
v6-1	391 (v6), 408 (v6)	386 (v6), 380 (v6)
v6-2	381 (v6), 409 (v6)	414 (v6), 405 (v6)
Parent	364 (V6)	
Parent		362 (v6)

[1] Genotype: *V6* = *UhAvr6* dominant allele, *v6* = uhavr6 recessive allele

Table 5.3 Results of different marker analyses on the pools and progeny.

Approach	No. of primer pairs tested	Primers that work in U. hordei	Polymorphic in pools and parents	Linked to UhAvr6 or uhavr6
SSRs (U. maydis)	115	56 (50 %)	10	0
SSRs (U. hordei)	55	55	3	0
AFLP	60	60	2	0
RAPD	> 200	150	10	0

CHAPTER 6

General discussion and future perspectives

6.1. General discussion

The main theme of my dissertation research was to study the avirulence gene *UhAvr1* and other effectors from *U. hordei*. The goal of my research was to extend our knowledge of effector-triggered immunity in plants to include an interaction between a basidiomycete pathogen and a monocot host, and to further our knowledge of fungal effectors in disease establishment and defense induction. To this end, research was conducted towards five objectives (Chapter 1).

The first objective was the identification and characterization of the *Ustilago hordei* avirulence gene 1 within the *UhAvr1* locus, and possibly *UhAvr6*, to improve our knowledge of effector-triggered immunity in plants. I have identified *Uh10022* as the *UhAvr1* gene (Chapter 2) which was previously mapped to an 85 kb genetic segment through a genetic marker-based approach (Linning, et al. 2004). UhAVR1 is a relatively small, predicted secreted protein with no homolog with known function in the public databases. In this regard, *UhAvr1* is similar to most of the identified fungal avirulence proteins, which are also usually small proteins, possesses an N-terminal secretion signal peptide and lack homology to sequences available in public databases (Dean, et al. 2005, Fudal, et al. 2007, Gout, et al. 2006, Rep, et al. 2005). In the analysis of this gene, I showed by both deletion of the C-terminal end of UhAVR1 and complementation approach, that this gene is responsible for inducing resistance in the barley cultivar Hannchen having resistance gene *Ruh1*.

The expression of the *UhAvr1* gene was not detected in any conditions tested in this study. The reason for this result may be that the expression of this gene is highly regulated and might be expressed at a very low level only during the early stage of infection. But obtaining enough fungal material from an early infected plant is a challenge. The deletion of this gene from an avirulent strain did not affect the disease on a barley cultivar not having the cognate *Ruh1* gene this suggest that this gene might be dispensable for infection or that some other genes had similar function. Another explanation is that this gene might cause only subtle variations in virulence that could only be detected in a population, or that the gene might have additive effects

on virulence. The virulence was measured by the number of diseased plants out of a total of inoculated plants; this is not a sensitive assay.

I did not succeed in the isolation of the *UhAvr6* gene using DNA marker-based approaches (Chapter 5). However, this study made it clear that the different virulent and avirulent strains of *U. hordei* that were used in this study for finding markers linked to the *UhAvr6* gene, were similar to each other at the DNA level at this locus. It is possible that small variations could be responsible for the avirulence/virulence allelic differences. A single base pair mutation, as shown for several other *Avr* genes from different pathogens, might be responsible for changing the avirulence to virulence phenotype at this locus (Joosten, et al. 1994, Parlange, et al. 2009, Schurch, et al. 2004, van Esse, et al. 2007, Westerink, et al. 2004). The molecular marker-based approaches used in this study were not very efficient for revealing point mutation.

The second objective dealt with how *U. hordei* overcomes *Ruh1*-triggered resistance in barley. To overcome ETI due to the recognition of avirulence proteins by plant R proteins, natural selection pushes the pathogens to escape recognition by the host (Dawkins and Krebs 1979). ETI can be overcome by several mechanisms to avoid recognition at the gene level by point mutations, frameshifts, recombination, deletions, gene duplications, gene disruption by transposable element (TEs) and acquiring novel genes. Deletion of effectors is feasible if their functions are dispensable or when genes with redundant functions are available in the genome. The loss of indispensable genes can also be countered by acquiring novel genes with a redundant function through horizontal gene transfer. Insertions of TEs into a promoter element or into the ORF of the gene can change gene expression, or the expressed protein itself, respectively (Daboussi and Capy 2003, Ganko, et al. 2003, Hua-Van, et al. 2002, Kang, et al. 2001). The influence of repetitive elements can result in deletions, inversions, duplications and translocations based on the relative orientation and location of the repeat with respect to the target gene (Daboussi and Capy 2003, Hua-Van, et al. 2000, Khang, et al. 2008, Kim, et al. 1998, Nitta, et al. 1997). I have shown in Chapter 2 that the *UhAvr1* gene is embedded in transposons and repeats and that the activity of TE is responsible for the inactivation of the gene. In contrast to gene deletion, the mutation in the avirulence gene caused by TE activity resulted in a non-functional allele, that could be beneficial for *U. hordei* as it is preserved in the genome and can be available for reversion once the selection pressure from the host is over (Stergiopoulos, et al. 2007). Several bacterial, fungal and oomycete avirulence genes have been shown to be

inactivated by TE activity (Orbach, et al. 2000, Rep, et al. 2004, Houterman, et al. 2008, Kim, et al. 1998, Rehmany, et al. 2003, Bohnert, et al. 2004, Farman, et al. 2002, Farman and Leong 1998, Kang, et al. 2001, Luderer, et al. 2002b).

After entry to the host cell or apoplast, the interaction between the AVR and R proteins can be either direct or indirect. It has been shown that effector proteins that bind directly to R proteins contain two distinct effectors and avirulence domains enabling them to change their binding domains without changing their effector function (Wang, et al. 2007). Such proteins are subject to diversifying selection which generates highly divergent alleles by gene duplication followed by point mutation as shown for the *AvrL567* locus in *Melmpsora lini* (Dodds, et al. 2006, Ellis, et al. 2007a, Wang, et al. 2007), *ATR1*^{NdWsB} and *ATR13* in *Hayaloperonospora arabidopsidis* strains (Deslandes, et al. 2003, Jia, et al. 2000, Rehmany, et al. 2005) and *Avr1-b1* in *Phytophthora sojae* (Dou, et al. 2008a). In contrast, indirect recognition of effectors by R proteins results in selection on AVR effector functions and therefore purifying selection (Bent and Mackey 2007). Under this regime, these effectors will favour mutations that render these proteins either non-functional or non-expressed or removal from the organism (Armstrong, et al. 2005, Rooney, et al. 2005). As revealed by the monomorphic nature of UhAVR1 among several field isolates and its inactivation by TE activity in virulent isolates (Chapter 2), I speculate that the interaction between UhAVR1 and RUh1 is indirect. However, this needs to be verified experimentally. The isolation of the *Ruh1* gene from barley could shed light on whether the interaction is indirect or direct.

The third objective was focused on obtaining an understanding of the evolutionary pressures acting on the *UhAvr1* locus. The region containing this locus is syntenic to *U. maydis* cluster 19, harbouring many predicted effectors (Kamper, et al. 2006) and a similar region in *Sporisorium reilianum* (Schirawski, et al. 2010). The synteny is highly conserved between the three organisms over the genes flanking the cluster on each side. Most of the predicted secreted proteins in the *U. maydis* cluster are represented by at least one homolog in *U. hordei*. However, in contrast to *U. hordei*, the gene families are much more conserved between the two corn pathogens. It can be assumed that this cluster is generally important for all three organisms. *U. hordei* contains several unique genes at this locus, and several genes are transcriptionally inverted compared to the two corn pathogens. In this regard, the region is much more diverged in *U. hordei* compared to the other two organisms possibly because the host is different; *U. maydis*

and *S. reilianum* infect corn while *U. hordei* infects barley. The region is enlarged in *S. reilianum* and *U. hordei* in comparison to *U. maydis*; both *U. hordei* and *S. reilianum* have more genes in this region and the intergenic region is enlarged compared to *U. maydis* (Schirawski, et al. 2010). In *U. hordei* the region is saturated with repeats and transposable elements which may be involved in the re-arrangement of this region and breaking of avirulence on barley cultivars containing the *Ruh1* gene. The co-localization of repeat and TE elements with *Avr* genes is a common feature in several pathogens and appears to supply a common mechanism used by pathogens to overcome host ETI (Orbach, et al. 2000, Rep, et al. 2004, Houterman, et al. 2008, Kang, et al. 2001, Kim, et al. 1998, Luderer, et al. 2002b, Rehmany, et al. 2003). Given the overall conservation in three organisms, it is likely that these genes in a cluster-like setting were obtained from a common ancestor before speciation but that each pathogen evolved independently in response to their specific biotropic life styles and hosts. In *U. maydis* and *S. reilianum*, there are several alleles of the same secreted proteins at this locus which indicate that diversifying selection might have acted upon this locus as a result of direct interaction between the effector and corresponding resistance gene. In these species, diversification might have acted on these genes to overcome host recognition. This is a bit surprising as no effector-*R* gene interaction has been shown to exist for *U. maydis* and *S. reilianum* with corn. I speculate from the gene duplications found at this locus in the two corn-infecting smut that there may have been effector-*R* gene interactions sometime in the past. It will be interesting to test a series of *U. maydis* and *S. reilianum* isolates on corn and other related wild species of corn and grasses to find out if some of these effectors are avirulence factors.

The fourth objective was geared towards gaining insight to the potential repertoire of small secreted proteins (effectors) of *U. hordei* likely involved in virulence and avirulence towards the barley host. My hypothesis was that secreted proteins that have functions in host cells, i.e effectors, would be direct targets of evolution. In biotrophic pathogens such as *U. hordei*, these effectors likely reprogram the host cells for their own benefit. My approach was to mine the genome of *U. hordei* to obtain its secretome and to compare it with other publicly available genomes of basidiomycetes and other fungal pathogens (chapter 3).

The CSEPs (candidate secreted effector proteins) that were identified in *U. hordei* in this study, were either hypothetical or did not have a known function. More than 50% of the CSEPs have four or more cysteine residues and several have a cysteine-content and spacing

characteristic for secreted apoplastic avirulence proteins. The percentage of CSEPs that have orthologs in related smut was very low as compared to non-secreted proteins this suggest that these proteins evolved at a faster pace compared to the rest of the proteome. In oomycete pathogens such *Phytophthora sojae, P. ramorum* and *P. infestans*, only one quarter of the effectors have orthologs in other species (Tyler, et al. 2006). The secreted effectors of *Blumeria graminis* f. sp. *hordei* also have very low numbers of orthologs in two other powdery mildew fungi infecting dicotyledonous plants (Spanu, et al. 2010). The high rate of sequence diversification, gene duplication and genome rearrangement in the effector-encoding genes may be the result of the ongoing molecular arms race between the pathogen and the host. This may also be responsible for the lack of orthologs in other pathogens. Effector-encoding genes are also usually located in highly flexible regions of the genome such as telomeres or embedded in transposable elements and thus can easily mutate and overcome the R-mediated resistance.

In *U. maydis*, 17 clusters of predicted secreted protein-coding genes were identified which were distributed randomly over the genome and comprised 26% of the complement of predicted secreted proteins (Kamper, et al. 2006). Genes in a variety of these clusters were induced during infection and deletion of eight of them affected pathogenicity on corn seedlings (Kamper, et al. 2006, Schirawski, et al. 2010). The induction of some cluster genes was also tissue specific and the effects of deleted clusters were variable depending on the host tissue infected (Skibbe, et al. 2010). In a comparative genomic study between *U. maydis* and *S. reilianum*, 43 clusters in which genes diverged between these species, were identified and 61% of the genes encoded predicted secreted proteins (Schirawski, et al. 2010). Analyzing the predicted secreted proteins of *U. hordei,* 62 clusters were identified (Chapter 3). The majority of the genes in these clusters encoded hypothetical, conserved hypothetical or *Ustilaginaceae*-specific conserved hypothetical proteins and some were very similar to gene clusters in *U. maydis* and *S. reilianum* (Kamper, et al. 2006, Schirawski, et al. 2010). In most other completed fungal genomes, no cluster arrangements of CSEPs were identified (Dean, et al. 2005, Spanu, et al. 2010). Gene clusters in other fungi are usually involved in sexual reproduction, or biosynthesis or degradation of secondary metabolites (Gardiner, et al. 2004, Kupfahl, et al. 2006). In several ascomycetes such as *Fusarium solani*, *F. oxysporum*, *Alternaria alternata* and *Cochliobolus* spp., genes with putative virulence functions are co-located on the same

chromosome, sometimes they interspersed with repeats, transposable elements, or other genes (van der Does and Rep 2007).

It is not understood how genes involved in virulence or avirulence evolved in clusters in fungal pathogens. Gene clusters in fungi with unknown functions are extremely unusual. Clustering may be necessary for co-regulation of the genes, especially if several virulence genes are required for infection of a particular plant host (van der Does and Rep 2007). It might be true for at least some of the clusters in *U. maydis* as genes in these clusters co-induced in the host (Kamper, et al. 2006, Schirawski, et al. 2010). The alternative hypothesis, suggested by van der Does and Rep (2007) is that virulence genes may appear at random positions in the genome and then cluster together as a result of random gene shuffling due to a strong selective advantage. For example, gene duplications that have been observed in several clusters in *U. maydis, U. hordei* and *S. reilianum* might be the result of direct interactions with *R* genes of the host as a mechanism to avoid host recognition.

In objective five, I developed an efficient *Agrobacterium*-mediated transformation (AMT) system for *U. hordei* for transferring large fragments of genomic DNA (chapter 4). Prior to this work, the transformation was carried out by electroporation of partial protoplasts, a procedure which is poorly reproducible and inefficient. This AMT method will facilitate greater understanding of the pathogenicity in *U. hordei* by allowing gene complementation especially through the transfer of large DNA fragments containing genes with regulatory sequences necessary for gene function, or complete gene clusters, to assess the location of specific functions/genes on such genome-size fragments as represented by Bacterial Artificial Chromosome (BAC) inserts. As shown, *U. hordei* harbours several gene clusters coding for predicted secreted proteins that could be effectors acting during host interactions, similar to those clusters described in *U. maydis* (Kämper et al. 2006, Chapter 3).

In conclusion the work described in this thesis chronicles the isolation of the *UhAvr1* gene and the characterization of the locus through comparison to the syntenic locus in *U. maydis*. New tools for functional genetic analysis have also been developed. Computational mining of the sequenced genome has yielded an inventory of a complement of potential secreted effector proteins. However, my work has generated new, exciting questions that should be addressed in future work. Below, I suggest some follow-up experiments that could make significant contributions to the field.

6.2. Future perspectives

6.2.1. Localization of UhAVR1

Fluorescent protein tagging is a powerful tool in molecular and cell biology used to monitor subcellular activities such as protein-protein interaction, protein localization, gene expression, protein movement, cell division and vesicle and organelle trafficking in living cells (Leffel, et al. 1997, Takemoto, et al. 2003). Chimeric constructs of the green fluorescent protein (GFP) or mCherry and the UhAVR1 protein will be helpful in investigating its localization during the *U. hordei* infection process (Khang, et al. 2010, Kale, et al. 2010, Kemen, et al. 2005). Effector proteins from fungal pathogens act either in the apoplast or after cell entry in the cytoplasm or nucleus (Bryan, et al. 2000, Dodds, et al. 2004, Dodds, et al. 2006, Jia, et al. 2000, Orbach, et al. 2000, Staskawicz, et al. 2001). This can be done by fusing *UhAvr1* to fluorescent protein sequences at either the N-terminal or C-terminal extension and then transferring the constructs into the *Uh10022* deletion strain (Uh364-Δ*10022*). These transformant strains should then first be tested on barley cultivar Hannchen for their ability to complement the mutation and show that the chimer still retained its avirulence function. They can then be inoculated on resistant (Hannchen) and susceptible (Odessa) barley cultivars and monitored throughout the infection cycle of *U. hordei* at the tissue and cellular levels by using fluorescent and confocal microscopy. This will enable visualization of the expression and location of the UhAVR1 protein during both compatible and incompatible interactions.

In addition to fluorescent protein tagging, immunochemistry can be used to obtain a more precise localization of UhAVR1. Immunochemistry utilizes antibodies to visualize antigens in sections of tissue using light or electron microscopy (Walker, et al. 2001)**.** For visualization, specific antibodies must be produced and incubated with pre-prepared embedded sections of cells and tissues. This immunochemistry analysis will also be helpful in investigating whether the *UhAvr1* gene is constitutively expressed or expressed under specific conditions. This analysis should also allow to determine the extent to which UhAVR1 is secreted into plant tissue during infection. If these experiments prove that UhAVR1 is indeed secreted into the host plant, it will then be highly interesting to unravel the mechanism by which host barriers are crossed.

6.2.2. Novel host protein interactions with UhAVR1

The interaction between UhAVR1 and the host target(s) will provide more insight into the infection process of barley by *U. hordei*. The yeast two-hybrid assay system could be used to find fungal and barley proteins interacting with UhAVR1. It is an easy, comparatively quick and flexible technique used for protein-protein interaction studies. This technique is based on the transcriptional activation of GAL4, a modular protein that requires the interaction of two domains with different specific functions (a DNA-binding domain, BD and an activation domain, AD). The *UhAvr1* gene can be fused with the *Gal4* BD and transfected into a receptor yeast strain harbouring the upstream activating sequences (UAS) and reporter gene which results in a hybrid bait protein. A barley cDNA library in hunter specific yeast two hybrid vectors with GAL4 AD is available from our collaborator Dr. R. Hückelhoven (J-L. University, Giessen, Germany) and will be transfected into the yeast strain containing the *UhAvr1-GAL4* construct. Colonies expressing the reporter gene will be those where UhAVR1 interacts physically with the cognate protein coded by cDNA clones from barley. It might be possible to find the corresponding *Ruh1* gene in barley if its product interacts with UhAVR1 directly. If the interaction is not direct, the UhAVR1 trap will identify other host targets. The *Ruh1* gene has been genetically identified to a 300 kb region on the short arm of chromosome 1. A more efficient approach might be to identify cDNA clones located in that region such as *R*-gene like genes, and apply them to the UhAVR1 trap assay. Many other avirulence proteins that are secreted into the host and which interact directly with R proteins of the host or other host factors have been identified using this system. It is possible that no interaction is found between UhAVR1 and barley proteins coded by the cDNA library. A complementary approach would be to transfer a cDNA library from *U. hordei* into a yeast strain transformed with the *UhAvr1*-bait-construct. In this way, interactions between UhAVR1 and possibly other proteins of *U. hordei* could be found. Positive cDNA clones of barley and *U. hordei* can be sequenced to identify the genes. Further molecular characterization of the interacting proteins can be validated *in planta* by bimolecular fluorescence complementation studies.

6.2.3. Function of UhAVR1

I have shown that UhAVR1 did not exhibit virulence functions towards barley cultivars Odessa and Hannchen by inoculating them with *U. hordei* strains where both mating partners carried the deletion for C19A2 (chapter 2). However, this experiment should be repeated based on our later finding that in the virulent strain Uh362, this region is translocated to another part of the genome. Truely isogenic strains should be created by crossing the generated *UhAvr1* 3′-deletion mutant in the Uh364 (*MAT-1*) background with the avirulent parental strain Uh365 (*MAT-2 Avr1*) and selecting *MAT-2* progeny on carboxin; which should have then the *UhAvr1* 3′-deletion as well. Work in Bakkeren laboratory is also ongoing to find the location of the translocated fragment in the virulent parent.

More work is required to establish the molecular function of UhAVR1. Future efforts should focus on gaining insight into the intrinsic function of UhAVR1 in pathogenicity. This can be done in several ways. One approach would be to inoculate barley cultivars Odessa (*ruh1*) and Hannchen (*Ruh1*) with both the *UhAvr1* deletion mutants (Uh364 Δ*UhAvr1*) and *U. hordei* wild type (Uh364 *UhAvr1*) strains followed by inoculation with a secondary barley pathogen such as *Blumeria graminis* f. sp *hordei* (*Bgh*). *B. graminis* f. sp. *hordei* is a barley pathogen that penetrates epidermal cells and whose subsequent infection process can be easily monitored. If UhAVR1 suppresses the host defense response, the secondary pathogen would produce increased severity of symptoms on Odessa when pre-inoculated with the wild type strain as opposed to the deletion mutant strain. On the other hand, on Hannchen, UhAVR1 should elicit a host defense and as a result the *B. graminis* f. sp. *hordei* should produce decreased symptoms on barley that is pre-inoculated with the wild type strains compared to mutant strains. An alternative to this approach would be the co-bombardment or agro-infiltration of resistant and susceptible barley coleoptiles with a fluorescent marker such as GFP as a reporter gene plus UhAVR1, followed by assays and testing for reduced reporter gene expression as a result of cell death or HR (Dong, et al. 2009, Rehmany, et al. 2005). Another approach would be to clone *UhAvr1* and a fluorescent protein-coding gene such as luciferase in different cereal-expressing plasmids and transform barley protoplasts with the two plasmid mixed in equal ratio and monitor cell viability. This approach has been used successfully to identify virulence functions for three avirulence genes from *Magnaporthe oryzae* by transferring them into rice protoplasts (Yoshida, et al. 2009). Finally, additional work is ongoing in the Bakkeren laboratory, using a cereal-adapted

Pseudomonas species for type III secretion-mediated translocation of the UhAVR1 protein into barley leaves and monitoring for suppression or induction of the defense response in susceptible and resistant barley cultivars, respectively.

6.2.4. Cloning of other *U. hordei* virulence and avirulence genes

For the cloning of *UhAvr6* and other avirulence genes (*UhAvr2* and *UhAvrx*) which segregate in the two populations in Bakkeren laboratory, a more targeted approach should be used. The majority of known avirulence factors from fungal and oomycete pathogens are secreted into the host (Ellis, et al. 2007b, Kamoun 2007). To identify genes showing polymorphisms correlated with avirulence functions, primers should be designed 1 kb upstream and downstream of all genes coding for the predicted secreted effector proteins in *U. hordei* (Chapter 3). PCR amplification will reveal presence/absence and/or difference in size of the amplified fragments. The use of these primers to screen the parents and bulked segregant pools could clearly indicate any marker linked to these avirulence genes. Any linked marker could be further verified on individual progeny of the mapping population. The same PCR products could be used for Targeting Induced Local Lesions IN Genomes (TILLING) studies to look for point mutations (Comai, et al. 2004, Rakshit, et al. 2007). An alternative would be the deletion of all the clusters of predicted secreted protein-coding genes that have been identified in this study (Chapter 3). These deletion mutants could be subsequently inoculated on the set of differential barley cultivars available. *UhAvr1* is located in a cluster that has the least-conserved genes between *U. hordei* and *U. maydis*. All clusters representing such diverged genes should be targeted first. Another strategy would be to employ an association mapping approach (Yoshida, et al. 2009). This would entail sequencing of the genomes of different strains, especially from the other parents of the populations, by next-generation sequencing technologies. Variation in predicted secreted effector proteins/genes, such as organization, deletions and single nucleotide polymorphisms (SNPs), can be revealed and correlated to virulence phenotypes (Yoshida, et al. 2009).

To reveal candidate secreted effector proteins (CSEPs, Chapter 3) with virulence or avirulence functions, it should first be confirmed whether these candidates are secreted. This can be done using the Yeast Secretion Trap system (Lee, et al. 2006). To assess whether effectors are induced during biotrophic growth, which has been shown to occur for most of effectors, *U.*

hordei microarrays or RNA sequencing could be employed. Functional analysis of these effectors could then be undertaken by transient expression in different barley cultivars using co-infiltration of effector genes together with marker genes such as *GUS* or *GFP* as discussed, above (Rehmany, et al. 2005). Virulence functions of these CSEPs, such as the suppression of host defense responses in susceptible plants or the elicitation of the host defense in resistant plants, can be carried out using secondary pathogens as described above. For protein localization, fluorescent protein tagging or immuno-chemistry could be used, as previously described.

6.3. Conclusion

The identification of *UhAvr1* has broadened our knowledge of how pathogens overcome resistance in host plants. In particular, *UhAvr1* is located in a repeat-and transposon-rich genomic environment prone to variation and under selection pressure. This region is syntenic to a similar region in closely-related basidiomycete smut fungi. Much more work is required to fully understand the intrinsic virulence function of this effector in host infections. To my knowledge, this is the first *Avr* gene isolated from a basidiomycete pathogen infecting cereal crops and as such can serve as a stepping stone to further our knowledge of plant-microbe interactions. The isolation of the corresponding *R* gene from barley using the cloned UhAVR1 effector will provide a potential new source for crop resistance. In my dissertation, I also exhaustively identified other candidate effector genes in this fungus which can be targeted in future studies. The virulence or avirulence functions of these effectors can be investigated by several approaches as suggested in the future perspectives section. This could lead to the isolation of other corresponding *R* genes, allowing for *R* gene pyramiding and increasing general crop resistance. The research tools developed in my work will prove helpful in the study of related pathogens.

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