IDENTIFICATION OF WHEAT LEAF RUST (*Puccinia triticina*. ERIKS.) GENES EXPRESSED DURING THE EARLY STAGES OF INFECTION

by

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B.S., Universidad Nacional de Colombia, 1997
M.S., Universidad Internacional de Andalucia, 2003

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Plant Pathology
College of Agriculture

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Manhattan, Kansas

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Abstract

In Kansas, wheat (*Triticum aestivum* L.) is severely affected by the biotrophic fungus *Puccinia triticina* (leaf rust). Although resistant varieties have been developed, the fungus tends to overcome new sources resistance very quickly. Plants have evolved a single gene (R genes) defense network that can recognize specific pathogen effectors (Avr), in a gene-for-gene manor. In rusts, effectors are secreted proteins responsible for inducing the uptake of nutrients and inhibit host defense responses. Identification of secreted proteins during the infection may help to understand the mode of infection of *P. triticina*. Little is known about molecular interactions in the pathosystem wheat-leaf rust and no Avr genes from cereal rusts have been cloned. In order to understand pathogenicity in leaf rust and generate new alternatives for disease control, the goal of this research is identify *P. triticina* secreted proteins from a collection of expressed genes during the infection, and to characterize putative Avr function for three candidates. From 432 EST's derived from haustoria and infected plants, fifteen secreted proteins were identified and 10 were selected as potential avirulence candidates. Pt3 and Pt 51 are two *P. triticina* (Pt) candidates expressed specifically in the haustoria and encode small cysteine-rich secreted proteins. Eight candidates are expressed at early stages of infection, during spore germination and 6 days after inoculation. They are small-secreted proteins. None are repetitive elements or have nuclear localization signals. They also do not share a conserved motif with known filamentous fungus Avr proteins. Five candidates are novel proteins, two have similarity with predicted proteins, one is homologous with Hesp-379-like protein, one is homologous with superoxide dismutase, and one has a cell glucanase predicted function. Pt3, Pt12 and Pt27 were tested by transient expression experiments using co-bombardment with GUS into leaf rust

resistant isogenic lines. Reduction in the expression of reporter gene GUS co-expressed with Pt27 indicates a potential avirulence factor for *Lr26* in wheat.

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Dedication

Dedicated to the queens: *Inés Narvaez Tobar* and *Blanca de Segovia Benavides*

Chapter 1 - **Identification of haustorium specific genes of wheat leaf rust (*Puccinia triticina*. Eriks.) that are expressed during early stages of infection.**

Plants provide an important food supply for animals and humans, and cereals provide the primary source of carbohydrates for most diets around the world. The forecasted human consumption of wheat (*Triticum aestivum* L.) is calculated to be 456 million tones for the year 2009 (FAOSTAT, 2009). Wheat is also important in animal feed and in industrial uses such as ethanol production. World production for wheat in the year 2009 was estimated at 24.98 billion bushels (656 million tones) and the U.S. was ranked fifth among the top world wheat producers with 2.2 billion bushels. U.S. is a leading wheat exporter world wide (USDA, 2009) and among the states, Kansas leads production with 360.8 million bushels (USDA, 2009).

Wheat is a monocot plant from the order of Poales, family Poacea and genus *Triticum*. Wheat species are classified according to the grain color (red, amber and white), the vernalization requirements (winter and spring), or the gluten content (hard and soft). For example; "hard red spring" is mainly used for bread flour or bread and hard baked goods, and "soft red winter" is used better for cakes, pies, biscuits and muffins (Cook and Veseth, 1991). Hexaploid wheat (*T. aestivum*) is the most widely cultivated species, although the tetraploid species, *T. durum,* is also cultivated (Cook and Veseth, 1991).

Growth and development of wheat is limited by abiotic environmental factors such as temperature, daylight length, water availability and soil conditions (e.g., salinity, acidity, alkalinity and aluminum toxicity). Wheat is also affected by biotic factors such as insect pests

and diseases. A wide range of pathogens can infect wheat and resistant wheat varieties provide an efficient strategy to prevent crop losses. Still, some of the pathogens are very persistent. For example, wheat leaf rust (*Puccinia triticina* Eriks) has been the major pathogen responsible for disease losses over the last 20 years in Kansas (Appel, *et al*. 2009). Although chemical control can be applied, it represents a significant increase in the cost of wheat production so the most desired method of control is the use of varieties with tolerance to abiotic stress, or resistance to insects and diseases.

Puccinia triticina belongs to the kingdom of Fungi, the phylum Basidiomycota, class Urediomycetes and order Uredinales. It is an obligate biotrophic parasite that is macrocyclic. The sexual cycle requires a secondary host *Thalictrum* spp., but the pathogen can skip the sexual cycle and replicate by an asexual cycle. Although the secondary host is not present in U.S., there are many *P. triticina* races with virulence to different varieties (Kolmer *et al*, 2007). The complete life cycle (Figure 1) produces five different spore types; urediniospores (dikaryotic), teliospores (dikaryotic), basidiospores (monokaryotic), pycniospores (monokaryotic) and aeciospoeres (dikaryotic). Urediniospores on the wheat leaf surface will germinate under high humidity and temperatures ranging from 15 to 20 ˚C. After elongation, the germ tube will extend and recognize a stomata by thigmotropism and initiate the formation of appresoria, which allow *Puccinia* to penetrate into the host apoplastic region. The fungus will start formation of primary hyphae, which attaches to the mesophyll. Twenty four hours after inoculation, the haustorial mother cell is separated by a neck-like structure, penetrates the host cell and develops into the haustorial body (Voegele and Mendgen, 2003). The haustorium remains separated from the host cytoplasm by the extra haustorial matrix and a membrane derived from invaginated host plasma membrane. Secondary hyphae are produced and form additional mother cells and haustoria.

After host colonization and during plant senescence, teliospores are produced in the wheat epidermis that overwinter and survive very low temperatures. In the spring under humid conditions, the promycelium will form, then sterigma and subsequently basidiospores will be produced. After release, basidiospores will infect the secondary host (*Thalictrum* spp.). One week later, the pycnia and receptive hyphae are produced on the *Thalictrum* leaf surface. Pycniospores are transported by insects to reach the receptive hyphae in order to fertilize and form aeciospores in the lower side of the leaves. Finally, aeciospores re-infect wheat and produce urediniospores to complete the sexual cycle (Figure 1; Bolton *et al*, 2008). In the absence of *Thalictrum*, the asexual cycle will occur. After small brown pustule (uredinia) formation, urediniospores will be released and transported by wind and subsequently deposited either in the soil or to overwinter on the wheat leaf surface. Urediniospores will rehydrate and germination begins with the formation of a germ tube. If no stomate is found the germ tube will continue elongation until the nutrient reserve in the spore is exhausted. Otherwise, after stomata penetration and host colonization, the uredinia will form 6 to 10 days post inoculation, erupt from the epidermis, and release urediniospores (Fig 1; Bolton *et al*, 2008).

Types of resistance

During their life cycle, plants are subjected to biotic stresses from insects, nematodes, fungi, oomycetes, bacteria and viruses. To overcome plant defenses, a pathogen will apply sophisticated strategies including formation of structures for penetration, degradation of plant barriers (cell wall, cutin, callose depositons), production of toxins and inactivation of host defense mechanisms (Dixon and Lamb, 1990). Plants utilize defenses like cell wall reinforcements, pubescence, cuticle, and biochemical pathways in order to ward off an attack.

One of the plant defense pathways is based on recognition of an essential and conserved set of molecules that cannot be modified or eliminated without affecting pathogen fitness. These are called PAMPs (pathogen associated molecular patterns) and examples include the bacterial flagellin, lipopolysaccharides, cold-shock protein (CSP), elongation factor Tu (EF-Tu), fungal chitin, ergosterol and the oomycete elicitor INF-1 (Dangl and Jones, 2001). PAMP recognition by transmembrane receptors activates PAMP triggered immunity (**PTI**). Despite the diverse nature of PAMPs, these molecules activate similar sets of plant responses, which include MAP kinase signaling, transcriptional induction of pathogenesis-related (PR) genes, production of active oxygen species (AOS) and deposition of callose to reinforce the cell wall (Nurnberger *et al*, 2004). In *Arabidopsis thaliana*, the receptor FLS2 is a receptor-like kinase (RLK) that perceives a conserved amino terminus in flagellin. Flagellin is the main building block of the bacterial flagellum and functions in locomotion (Felix *et al*., 1999; Gomez-Gomez and Boller, 2000; Chinchilla *et al*., 2006). PTI is considered as a primary defense response against pathogen attack, nevertheless pathogens have evolved to evade PTI and cause disease.

Effector triggered immunity (**ETI**) is the second biochemical strategy for pathogen recognition and plant defense. ETI relies on the recognition of pathogen effectors by a host resistance (R) protein. Recognition triggers a rapid programmed cell death called the hypersensitive response (HR). In contrast to PTI, which perceives a highly conserved molecules from different pathogens, ETI provides a race specific resistance since it involves the recognition of a specific effector in a particular host genotype in its adapted pathogen (de Wit, 2007). For example RPS2, an *A. thaliana* R protein, specifically recognizes the AvrRpt2 protein secreted by the bacterial pathogen *Pseudomonas syringae* (Kunkel *et al*., 1993). Another example is the tomato R protein, Cf9, which recognizes the *Cladosporium fulvum* effector Avr9 (van Kan *et al*.,

1991). ETI is an stronger response compared with PTI (Jones and Dangl, 2006). Several changes occur in plant physiology as a consequences of R-Avr recognition. Plant hormones will accumulate (ethylene, salicylic acid, jasmonic acid), there is an oxidative burst caused by increasing levels of active oxygen species, pathogenesis-related genes (PR) are induced, and phytoalexins are synthesized (Blumwald *et al*., 1998; Scheel *et al*.,1998). These plant physiological modifications lead to restriction of pathogen colonization (Jones and Dangl, 2006).

Plant Resistance Proteins

Plants have classes of proteins that specifically recognize pathogen-associated proteins and induce a defense response. Six major classes of R proteins have been identified in plants (Martin *et al*., 2003; Staskawicz *et al*, 2001; Yun, 1999) and are placed into two major groups according to the domain organization:

1- The transmembrane and extracellular leucine-reach repeat (LRR) proteins, which are grouped into three subclasses, receptor-like proteins with extracellular LRR and transmembrane domain [TM] (**RLPs)**, extracellular LRR with TM domain and cytoplasmic kinase (**RLK**) and polygalacturonase inhibiting protein with cell wall LRR; (**PGIP;** Chisholm *et al*. 2006).

2- The nucleotide binding, leucine-rich repeat (NBS-LRR) proteins, which are predicted to be cytoplasmic. This family based on their N-terminal domain can be subdivided into coiled-coil (CC-NBS-LRR) and Toll-interleukin-1-like receptor (TIR-NBS-LRR),

Numerous R genes have been cloned. The first was not a typical R gene, as defined alone. The maize *Hm1* encodes an NADPH-dependant reductase that inactivates a toxin

produced by the pathogen *Cochliobolus carbonum,* the causal agent of leaf spot of maize (Johal and Briggs, 1992). The majority of the R genes cloned belong to the NBS-LRR class, which provides resistance to several plant pathogens including viruses, bacteria, filamentous fungi and oomycetes. The tobacco *N* gene confers resistance to *Tobacco mosaic virus* (Whitham *et al*., 1994). RPM1 protein in *A. thaliana* confers resistance to *Pseudomonas syringae* pv. *maculicola* (Grant *et al*., 1995). The rice protein, Pita, confers resistance to *Magnaporthe oryzae* (Bryan *et al*., 2000) and Rpi-blb1 from *Solanum bulbocastanum* confers resistance to *Phytophthora infestans* (Vleeshouwers *et al*., 2008).

In wheat and other cereals, 67 leaf rust (*Lr*) R genes have been assigned (McIntosh *et al*, 2010) and additional R genes are still under analysis. These genes were characterized in common hexaploid wheat, tetraploid durum wheat and some diploid wild wheat. Four leaf rust resistance genes had been cloned *Lr1, Lr10, Lr21* (Cloutier *et al*., 2007; Feuillet *et al*., 2003; Huang *et al* 2003), which encode for NB-LRR cytoplasmic proteins, and *Lr34* which resembles an adenosine triphosphate-binding cassette transporter (Krattinger, *et al*. 2009). There are also more than 60 genes that confer resistance to stem rust (*P. graminis*) (Sr). In barley (*Hordeum vulgare*), there are 15 genes for resistance to stem rust and one of these has been cloned. *Rpg1* was cloned and encodes a receptor like-kinase with two tandem protein kinase domains (Brueggeman *et al*., 2002). There are 43 stripe rust (*P. striiformis*) resistance genes that have been assigned in wheat and more than 102 have a temporary designation. The broad-spectrum stripe rust gene *Yr36*, was cloned in 2009 and it corresponded to protein with a kinase putative STAR lipid-binding domain (Fu *et al*., 2009).

Gene-for-gene theory

The "gene-for-gene" interaction was first described by Flor (1955) in the pathosystem flax (*Linum usitatissimum*)-flax rust (*Melampsora lini*), and a single protein in plants (R) recognizes a specific pathogen and produces a strong defense response. Flor also noticed that resistance was related not only to R genes, but also to genes in the pathogen (*AVR*) since the incapability to infect was inherited by the rust. These observations suggest a clear recognition of the pathogen molecule by the plant and today it is described as **ETI** (Jones and Dangl, 2006). *S*uccess in the invasion depends on the genotype of both. Flor developed a protocol to test resistant plants with different pathogen races and this systematic evaluation allowed him to correlate plant resistance with alleles for reduced infection in the pathogen. The gene-for-gene theory constitutes a pivotal concept for modern phytopathologists and has been reported between several hosts and different pathogens and pests (Martin *et al*., 2003).

Effectors in Prokaryotes

During early stages of infection, bacterial effector proteins are injected into the host cytoplasm and neutralize crucial intracellular pathways, promoting pathogenicity (Staskawicz *et al*, 2001). Bacterial effectors can function as inhibitors of defense responses, activation of plant transcriptomes and suppression of programmed cell death (Mudgett, 2005). *Pseudomonas syringae* is the most studied bacteria from which numerous effectors have been cloned. *P. syringae* secretes AvrPto which is an E3 ubiquitine ligase that interferes with the cell death defense response (Janjusevic *et al*., 2005). AvrPt2 (Kunkel *et al*., 1993) and AvrPm1 (Ritter *et al*., 1995) inhibit basal defense responses. *Ralstonia solanacearum* and the majority of *Xanthomonas* species contain the transcription-activator like (TAL) effector family that have

nuclear localization signals (NLS) and an acidic transcriptional activation domain (AAD). These effectors alter plant transcriptomes during pathogen infection and down-regulate plant defense responses. *Xanthomonas oryzae pv. oryzae* TAL effector PthXo1 specifically induces Os8N3, which promotes disease susceptibility (Yang *et al*., 2006). This knowledge about bacterial effectors leads to a better understanding of virulence mechanisms in prokaryotes and allows the generation of novel strategies for bacterial disease management.

Effectors in Eukaryotic Microorganisms

The majority of fungal and oomycetes effectors are small secreted proteins with unknown function. Exclusively for oomycetes effectors, the motif RXLR was identified near the N-terminus (Birch *et al.,* 2008). No common motif has been identified for filamentous fungus effectors. To date, thirty-two Avr have been cloned (Table 1), most of them by map-based cloning and reverse genetics (de Wit *et al*, 2009). The first oomycete effector, from *Phytophthora sojae* (root rot on soybean), is AVR1b. Recognition of AVR1b by RPS1b protein leads to the resistance response in soybean plants carrying the gene (Shan *et al*, 2004). Avr1b encodes a small secreted protein with 138 amino acids (a.a) and is thought to work as a cytoplasmic effector (Kamoun, 2007). Recently, three additional effectors have been cloned from *P. sojae*: *Avr1a, Avr3a* and *Avr3c* (Qutob *et al*, 2009; Dong *et al*, 2009). All encode small secreted proteins in a size ranging from 101 to 152 a.a. There is also a set of effectors cloned from *Phytphthora infestans*, the causal agent of potato blight disease: *Avr3a*, *Avrblb1* and *Avrblb2*. AVR3a is a small secreted protein (147 a.a), which was identified by association analysis with polymorphisms in candidate genes (Armstrong *et al*., 2005). Recently it was demonstrated that AVR3a was able to suppress plant cell death by targeting plant E3 ligase

CMPG1, thereforemanipulating host defense (Bos *et al*, 2010). And *Avrblb1* and *Avrblb2* were identified from prediction of computationally effector candidate genes from *P. infestans* genome and allele mining with high-throughput *in planta* expression (Vleeshouwers *et al*, 2008; Oh *et al*, 2009). Both proteins are small cytoplasmic effectors which induce strong HR in potato plants carrying the cognate R gene.

The first filamentous fungal AVR gene cloned is *Avr9*, from the imperfect fungus *Cladosporium fulvum*, a hemibiotrophic tomato pathogen that colonizes the intercellular spaces in the host (van Kan *et al*., 1991). AVR9 is secreted into the apoplast and is recognized by Cf-9, an extracellular LRR-TM resistance protein. *Avr9* encodes a 63 amino acid protein with an N-terminal secretion signal peptide (18 amino acid) and six cysteine residues. To date, three more avirulence genes have been cloned from *C. fulvum*, and all of them are recognized by the cognate cellular LRR-TM resistance proteins: AVR2 (Dixon *et al*, 1996), a secreted-protein with 78 residues (8 cysteine) that it is expressed only in planta. AVR4 (Joosten *et al*, 1994) is a 135 a.a protein with a signal peptide and eight cysteine residues and it is expressed only during the infection. AVRECP2 encodes a 143 amino acid mature protein after cleavage of the signal peptide (Van Den Ackerveken *et al* 1993).

More *Avr* genes have been cloned from the ascomycete group. There are eight *Avr* genes cloned from the rice pathogen *Magnaporthe oryzae*: *AvrPita, AvrACE1, Pwl2, AvrPiz, AvrPiia, AvrPii* and *AvrPik/km/kp.* Seven encode for small proteins with an N-terminal signal peptide and their precise function in promoting disease is still unclear (Orbach *et al*, 2000; Bohnert *et al*., 2004, Kang *et al*,1995; Li *et al*., 2009; Yoshida *et al*, 2009). *AvrPita* has homology to fungal zinc-dependent metalloprotease. The extracellular pathogen *Fusarium oxysporum f. sp.*

lycopersici colonizes xylem vessels, and delivers effectors into the xylem. Three of them have been cloned. SIX1, which is recognized by R protein I-3, and renamed as AVR3, encodes for a small protein (284 a.a) with a signal peptide and eight cysteine residues. AVR3 seems to be required for virulence in tomato (Rep *et al*., 2006). SIX4, recognized by I-1 and renamed as AVR1, encodes small-secreted protein and in contrast with *Six1*, it is not required for full virulence in tomato. Two *Avr* genes were isolated from *Leptosphaeria maculans*, which causes blackleg disease in *Brassica* crops, using map based cloning. AVRLm1 is a 205 a.a protein with a SP and only one cysteine residue (Gou *et al*, 2006) and AVRLm6, is a small secreted protein (144 a.a), which is rich in cysteine residues (Fudal *et al* 2007). There is an exclusive set of effectors secreted by the haustoria of *Blumeria graminis* f. sp. *hordei*, a causal agent of powdery mildew in barley. AVRK1 and AVRa10, are small proteins (286 and 177 a.a; respectively) that have avirulance functions, but surprisingly they do not have the typical SP at their N-terminus (Ridout *et al*, 2006)

In basidiomycetes, *Avr* genes have been cloned from the flax rust, *Melampsora lini*. This obligate biotroph is characterized by the formation of haustoria, which it is an important feature with a role in nutrient uptake and effector delivery (Catanzariti *et al*, 2006; Mendgen and Hahn, 2002). The first flax rust *Avr* gene cloned, *AvrL567,* was isolated by using a suppressive subtractive hybridization cDNA library, and is enriched in rust genes expressed during the infection. AVRL567 protein is recognized by three different resistant genes (Dodds *et al*., 2004). In flax rust, the screening of haustorium-specific cDNA library lead to the identification of secreted proteins that co-segregate with avirulence in genetic crosses, so the cognate R gene was easily identified. In addition to the previously isolated AvrL567, new Avr genes; *AvrM, AvrP4*

and *AvrP123* were also identified and successfully cloned (Catanzariti *et al*., 2006). There is not an obvious function for them, since there are no similarities in the public database, but AVRP123 contains ten cysteine residues and has similarity with the Kazal family of serine protease inhibitors (Catanzariti *et al.,* 2006). Particularly for *AvrL567*, direct interaction with the corresponding resistant genes was demonstrated, using yeast two hybrid system (Dodds *et al*., 2006). Furthermore, the AVRL567 protein was crystallized and the three dimensional structure was solved (Wang *et al*, 2007). Remarkably, they found that amino acid changes in position 50 and 96 were critical for determining virulence or avirulence in different flax rust variants.

Interaction R-AVR proteins

After the gene-for-gene theory, the assumption was that AVR proteins from the pathogen directly interact with R proteins. Although direct interactions have been reported in some pathosystems such as rice-*M. oryzae* in Pita-AvrPita (Jia *et al*., 2000), flax-*M. lini* in L5L6L7-AvrL567 (Dodds *et al*., 2006) and *Arbidopsis thaliana-Ralstonia solanacearum* in Pop2-RRS1R (Tasset *et al*., 2010), no other direct interaction has been shown. The complexity of pathogenicity interaction can be explained by the **guard hypothesis**, which states that R proteins are monitoring host proteins, and detection of alterations caused by AVR proteins leads to a defenses response. This indirect interaction model includes an additional protein for the R-AVR interaction (Dangl and Jones, 2001). In the *P. syringae*-Arabidopsis interaction, AVRRpm1 interacts with RIN4 and activates resistance protein (Mackey *et al*., 2002). The guard hypothesis however, does not explain how the pathogen can overcome of resistance while the pathogen retains the AVR factor. Thus recognition specificity and virulence activity does not always

correspond each other. An alternative theory considers selective pressure over the guardian proteins, depending on the presence or absence of the corresponding *R* gene.

The **decoy model** suggests that the absence of the *R* gene will favor the evolution of host proteins that can mimic an effector target in the host, and act as a decoy for the pathogen. Such interaction leads to a resistance response (van der Hoorn and Kamoun, 2008). Decoy proteins cannot affect pathogen virulence. If the interaction occurs with a virulence effector that targets a specific host protein, then decoys can be molecular sensors of pathogen activity. To illustrate this, it was considered that the Ser/Thr kinase PTO was the host target in the pathosystem tomato-*P. syringae*, and PRF was the resistance protein acting as guardian for the action of AVRPto and AVRPTOB over PTO (van der Biezen and Jones, 1998). But recently, it was demonstrated that receptor like kinases CERK1, BAK1, EFR1 and FLS2, involved in PTI responses, were the real target of these effectors (Gimenez-Ibanez *et al*., 2009; Xiang *et al*., 2008), and PTO is acting as a decoy, which after a pseudo interaction, activates the PRF resistance response (van der Hoorn and Kamoun, 2008). The understanding of the interaction between the plant R protein and the pathogen AVR protein is a key question to develop new strategies of control and potentially durable resistance. Novel resistance strategies can be contemplated, such as the blocking of effector delivery machinery, modification of host targets in order to avoid effector function and the design of synthetic plant immune receptors to detect pathogen effectors (de Wit *et al*., 2009). Still, cloning *AVR* genes from eukaryote plant pathogens is a challenge task, considering the lack of common signatures, other than the presence of SP.

To date no cereal rust effectors have been cloned. Isolation of a rust AVR might provide the knowledge to generate new strategies of rust control. Little is known about *P. triticina* molecular biology, but recent publication of a draft genome sequence of *P. triticina* (Cuomo *et al.*, 2009) enables effector identification by comparative genomics within the *Puccinia* group. The biotropic nature of leaf rust complicates lab analysis, but previous approaches used by Catanzariti and collaborators on 2006 can be used. As in the pathosystem flax-flax rust, classical breeding information is available for the Lr genes identified, and the resistance response in a gene-for-gene manner enables the use of isogenic lines for effector analysis. So, the strategy of screening EST's from a haustorium specific library to generate secreted AVR candidates utilized by Catanzariti and collaborators (2006) can be implemented.

P. triticina is poorly characterized, and data about molecular biology in the interaction wheat-leaf rust is necessary. Success in the cloning of Avr genes from the biotroph flax rust encouraged us to implement the same strategy and generate information about the proteins secreted from leaf rust haustorium. This knowledge may lead to a better understanding of the pathosystem interaction. The hypothesis of this research relies on the assumption that leaf rust haustorium (as in flax rust) is enriched with secreted proteins, and some of them will have AVR function. A leaf rust haustorium specific cDNA library was made (Huang, 2006), as well as a cDNA library from wheat leaves infected with leaf rust. Analysis of the sequences generated will provide an idea about the proteins expressed in haustoria and during the infection, and enable the prediction of potential functions during plant infection. Identification of secreted proteins and evaluation for avirulence function in the candidates will test the hypothesis. Identification of candidates with Avr function will contribute to better understanding of cereal rust disease. The

purpose of this work is: 1-Characterize a cDNA haustorium specific library and infected plant ESTs and identify predicted secreted proteins from *P. triticina*; 2-Validate predicted secreted proteins as AVR genes.

Figure 1 Life cycles of *Puccinia triticina* adapted from the web page of Kolmer 2009, **http://www.ars.usda.gov/pandp/people/people.htm?personid=3094,.**

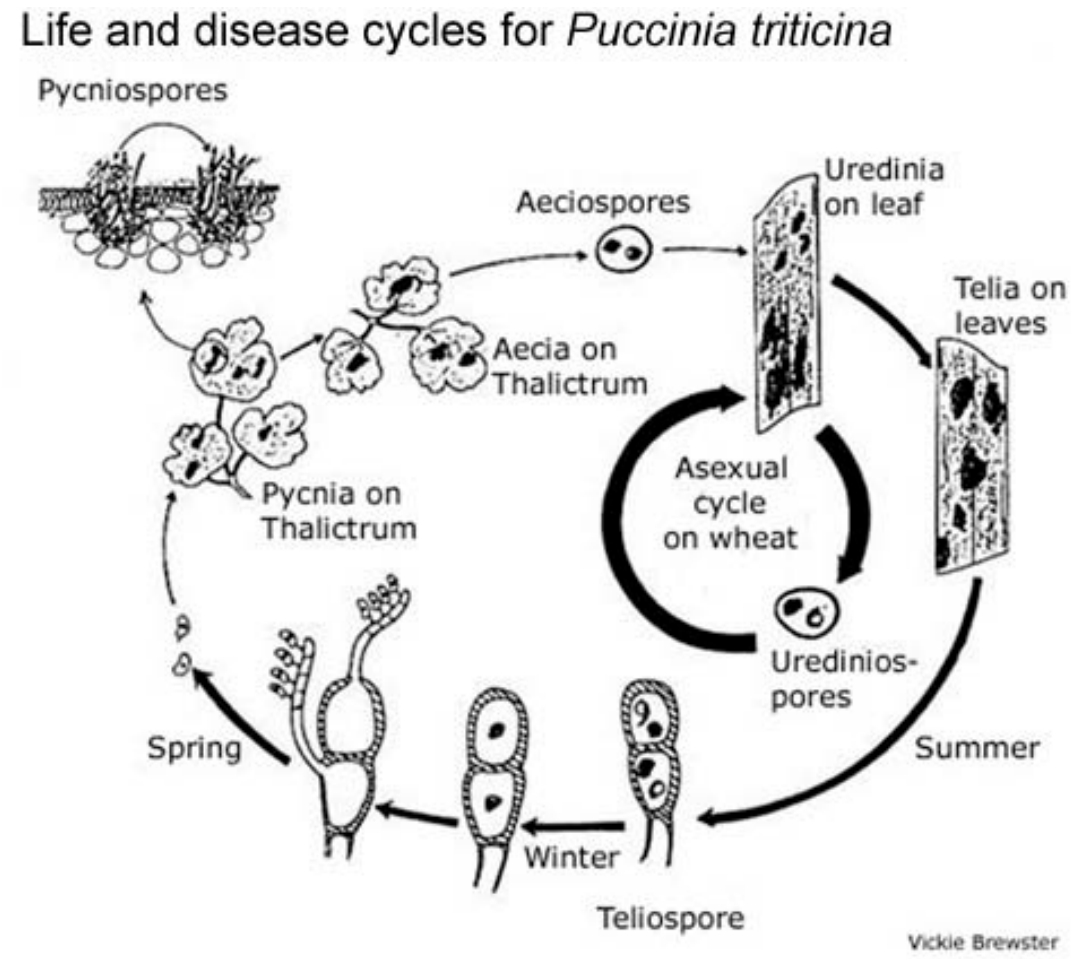

Table 1. Fungal and oomycetes effectors

	Organism	Name	a.a	SP Length	SP Score		Description	Reference
					Mean S	HMM		
FILAMENTUS FUNGI	*Cladosporium fulvum*	Avr2	78	20	0.959	1	Cystein-rich protein; Protease inhibitor. Inhibit Rcr3,Pip1, aleurain and TDI-65	Dixon *et al.,* 1996
		Avr4	135	18	0.909	1	Cysteine-rich protein. Contains CMB14 chitin binding domain. Protects cell wall from hydrolysis by plant chitinase	Joosten *et al*., 1994
		Avr9	63	23	0.966	0.999	Contains cysteine knot motif	Van Den Ackerveken et al. 1993
		AvrEcp2	165	22	0.833	0.989	Contains Even number of Cysteine	
	Magnaporthe oryzae	AvrPita1	224	16	0.921	0.949	Putative Metalloprotease	Orbach *et al*., 2000
		PWL 2	145	21	0.868	0.982	Glycine-rich hydrophilic protein	Sweigard *et al*., 1995
		Avr-Pia	85	19	0.881	0.995		Yoshida *et al*., 2009
		Avr Pii	70	19	0.927	0.943		
		Avr Pik/km/kp	113	21	0.823	0.988		
	Melampsora lini	AvrL567	150	23	0.64	0.966		Dodds *et al*., 2004
		AvrM	314	28	0.675	0.862	Cysteine-rich protein	Catanzariti *et al*., 2006
		AvrP123	117	23	0.93	0.955	Cysteine-rich protein	
		AvrP4	95	28	0.851	0.51	Cysteine-rich protein	
	Fusarium oxysporum f. sp. *lycopersici*	SIX1 (Avr3)	284	21	0.893	0.988	Xylem. 8 Cysteine residues	Rep *et al*., 2004
		SIX3 (Avr2)	163	19	0.935	0.999		Houterman *et al*., 2007
		SIX4 (Avr1)	242	17	0.784	0.993		
	Blumeria graminis f. sp. *hordei*	Avrk1	286	-	-	-		Ridout *et al.,* 2006
		Avr10	177	-	-	-		
	Rhynchosporium secalis	Nip1	82	22	0.827	0.999	10 Cysteine residues involved in intramolecular disulphide bonds	Rohe *et al*., 1995
		Nip2	109	16	0.902	0.982	6 to 8 Cysteine residues	

Table 1 *continued*..

	Organism	Name	a.a	SP Length	SP Scores		Description	References
					Mean S	HMM		
FUNGI	*Leptosphaeria maculans*	AvrLm1	205	22	0.827	0.999		Gout *et al*., 2006
		AvrLm6	144	20	0.902	0.982	6 Cysteine residues	Fudal *et al*., 2007
		AvrLm4-7	143	21	0.907	0.990	8 Cysteine residues	Parlange *et al*., 2009
OOMYCETES	*Phytophthora infestans*	Avr3a	147	21	0.984	0.999	Cell death suppressor by targetting E3 ligase CMPG1	Armstrong *et al*., 2005
		Avrblb1	152	21	0.919	1		Vleeshouwers *et al*., 2008
		Avrblb2	101	22	0.569	0.530		Oh *et al*., 2009
	Phytophthora sojae	Avr1b	138	21	0.832	0.998		Shan *et al*., 2004
		Avr 1a	120	23	0.850	0.999		Qutob *et al*.,2009
		Avr 3a	111	20	0.894	0.999		
		Avr3c	221	20	0.878	1		Dong *et la*. 2009
	Hyaloperonospora Arabidopsidis	ATR13	150	19	0.783	0.978		Allen *et al*., 2004
		ATR1	310	15	0.909	0.988		Rehmany *et al*., 2005

aa: Aminoacid Size; SP: Signal Peptide.

Chapter 2 - **Characterization of a *Puccinia triticina* haustoria derived cDNA library and identification of candidate effector genes expressed during the infection of wheat**

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Puccinia triticina Eriks is an obligate biotrophic pathogen that is the causal agent of leaf rust, also known as brown rust, in wheat (*Triticum aestivum* L.). Leaf rust is the most severe wheat disease in Kansas (Appel *et al*., 2009) and resistant varieties are used as an effective way of disease control. However, soon after the variety release, new races of rust arise and resistant varieties lose effectiveness. There is a need for new strategies of resistance and a deep understanding of the wheat-leaf rust interaction will allow the development of novel sources of disease control. Knowledge of the wheat-leaf rust pathosystem interaction is still limited. In susceptible varieties, urediniospores will germinate in the leaf surface and a germ tube will find a stomate, form an appressorium, and initiate infection. Following penetration, the fungus will form substomatal vesicles (SSV), primary hyphae, and a haustorial mother cell. After twenty-four hours the mother cell will be separated from the infection hyphae by a septum and produce haustoria, which are specialized infection structures that invaginate the host cell cytoplasm. Without disrupting the plant cell membrane, haustoria will begin to secret effectors that reprogram the host cell and allow the fungus to take up nutrients (Bushnell, 1972; Hann and

Mendgen, 2001; Voegele and Mendgen, 2003). Oomycetes and powdery mildews are other obligate biotrophs that also form haustoria. Thus, haustoria are critical for the infection process, and genes expressed therein have an important role in the biotrophic interaction.

Recent advances in the understanding of pathogenicity of plant microbes demonstrate that effectors are key players that can suppress or interfere with the host defense response (Solomon and Rathjen, 2010). Many of the characterized effectors share conserved or easily identifiable motifs which can be utilized by bioinformatic approaches. Members of the TAL (transcription-activator like) effector family in the plant pathogenic bacteria *Xanthomonas* spp. have nuclear localization (NLS) signals and an acidic transcriptional activation domain (AAD; Yang *et al*., 2006). Oomycete effectors have an RXLR domain in the N-terminal region following the signal peptide that is believed to be involved in translocation into the host (Kale *et al*., 2010). Unfortunately, characterized effectors from filamentous fungi do no share a common motif, nor do they have similarities with other characterized effector proteins. For instance, AVR_{k1} and AVR_{a10} from *Blumeria graminis* DC. Speer, do not have the typical N-terminus secreted signal peptide (Ridout *et al*., 2006).

Genetic and genomic approaches have been used to clone effectors with an avirulence function. Typically, map-based cloning is the preferred method, for example, *Pwl1*, *Pwl2* and *AvrPita* from *Magnaporthe oryzae* Couch were cloned using map based cloning (Valent *et al*., 1986; Sweigard *et al*., 1995; Orbach *et al*., 2000). However, many fungi are biotrophic in nature and genetic studies are very difficult and labor intensive. Rust Transferred Protein 1 from *Uromyces fabae* (UfRTP1; Hahn and Mendgen, 1997) and AVRL567, AVRP123, AVRP4 and AVRM from *Melampsora lini* Ehrenb. Lev (Catanzariti *et al*., 2006) were isolated by predicting secreted proteins from cDNA libraries made from haustoria. Yin and collaborators

(2009) generated ESTs from haustoria of stripe rust (*Puccinia striiformis* Westend), and fifteen genes were predicted to encode secreted proteins, but none have been verified as virulence factors. Unfortunately, no other Avr genes have been identified from rust pathogens. As sequencing and computational technologies have advanced, the generation of EST's now provide a rapid approach to find functional proteins and gene discovery (Hu *et al*., 2007).

There are genomic resources available for the cereal rusts. The *P. graminis tritici* genome sequence was released in 2007. The *P. triticina* genome was published in November 2009, and the *P. striiformis* genome sequence will be released in late 2010-2011 (Cuomo *et al*., 2009). As the sequence is characterized, proteins will be predicted and function can be assigned either biochemically or bioinformatically. Identification of proteins secreted by leaf rust during the infection will provide an insight into the wheat-leaf rust molecular interaction and have an impact in the development of disease control. The goal of this research was to create and characterize cDNA libraries from haustoria and infected tissue and identify candidates with avirulence function. Two libraries were made and putative secreted proteins were identified.

Materials and Methods

Plant material and Rust culture

Seedlings of the susceptible wheat cultivar 'Wichita' were grown in square pans (7.5 cm^2) containing Metro Mix 360 soil mix (Sun Gro, Bellevue WA) and grown in a growth chamber with 16 h day periods at a temperature of 21º C . Light levels were a flux density of 145 mol m-2 s-2. At the 2-3 leaf stage, plants were inoculated with 30 mg of uredineospores suspended in 2 ml of Soltrol 170 isoparaffin solvent (Chevron Phillips Chemical Co, The

Woodlands, TX). Spores were from *P. triticina* race PBJL (avirulent *Lr2a, Lr3ka, Lr9, Lr10, Lr16, Lr14a, Lr18, Lr24, Lr26, Lr30, /*virulent *Lr1, Lr2c, Lr 3a, Lr11, Lr17, LrB*). Inoculated plants were incubated overnight in a 100% humidity chamber at 18ºC. Plants were then transferred back to the growth chamber at the conditions listed above.

Haustoria Isolation, cDNA cloning and sequencing

The haustorial isolation followed the protocol developed by Hahn (1995). Heavily infected leaf tissue was harvested at 6 days post inoculation (dpi) and washed in deionized water. Eight grams of infected leaves were placed in 100 ml of ice-cold homogenization buffer (0.3 M Sorbitol; 20 mM MOPS, pH 7.2; 0.1% Bovine Serum Albumin (BSA); 0.2% 2-mercaptoethanol and 0.2 % PEG 6000) and homogenized in Waring Blender at maximum speed for 10-20 sec. The suspension was then filtered through a 20 µm nylon mesh, rinsed with homogenization buffer, divided into four-50 ml centrifuge tubes, and centrifuged in a JA-18 rotor at 5000-7000*xg* for 5-10 min. The supernatant was removed and pellets were re-suspended in 8 ml ice-cold suspension buffer (0.3 M Sorbitol, 10 mM MOPS pH 7.2, 0.2% BSA, 1 mM KCl, 1mM $MgCl_2$) and 1 mM $CaCl_2$). The preparation was centrifuged again at 5000-7000 x g for 10 min and the pellet resuspended completely in 4 ml suspension buffer. Two column void volumes of suspension were loaded onto a column with CNBr-activated sepharose 6MB beads (Sigma Aldrich, St. Louis, MO) and allowed to sit for 15 min. The column was overlayed with 2 column volumes of suspension, allowed to flow through and the rinse was repeated five times. The column outlet was closed and one void volume of suspension buffer was added and the column content was agitated by pipetting. The

sepharose beads were allowed to settle for 1-2 min, and the haustoria containing supernatant was transfered to a 1.5 ml tube. Haustoria were pelleted at 15,000 *x g* for 1 min in a microfuge.

Library construction and sequencing

Total RNA was isolated from the haustoria preparations using the RNeasy Plant RNA kit (Qiagen). cDNA was prepared and the library constructed using the SMART PCR cDNA Synthesis Kit (CLONTECH, Mt View, CA) and plasmids were transformed into DH5alpha *E. coli* cells. 4,128 colonies were isolated and grown in LB media (Sigma-Aldrich) containing 100 mg/L ampicillin. The library was sent to the Michael Smith Genome Sciences Centre in Vancouver, British Columbia, Canada. The library is a non-directional library and thus sequenced with three primers, S6Wu, TB24 and C21 using Sanger sequencing and Applied Biosystems 3730xl DNA analyzers (Appied Biosystems, Foster City, CA). Assembly of the reads into contigs was done with CAP3 algorithms (Huang and Madam, 1999).

Illumina Solexus Sequencing of cDNA from infected tissue.

A second cDNA library was sequenced using Next generation sequencing technology. Seedlings of the wheat cultivar 'Prairie Red' were grown and inoculated as before except they were inoculated with *P. triticina* race BBBD *(Lr1, Lr2a, Lr2c, Lr3a, Lr3ka, Lr9, Lr10, Lr11, Lr16, Lr17, Lr18, Lr24, Lr26, Lr30, LrB/ Lr14a*). At 6 dpi, heavily infected leaf tissue was isolated and total RNA was extracted using the *mir*Vana RNA kit (Ambion, Austin, TX, Cat number AM1561). Three-3 cm leaf segments were placed in a 1.5 ml centrifuge and ground to a

powder using liquid nitrogen and a plastic pestle. Seven hundred ul of Lysis/Binding buffer was added to the tube and vortexed. Seventy µl of miRNA Homogenate Additive was added, the tube vortexed and chilled on ice for 10 min. One volume of Acid-Phenol : Chloroform (Ambion Cat number AM9720) was added, the tube vortexed for 30 sec, and spun at RT at 10,000 *x g* in a microfuge. The upper phase was removed, placed in a new tube, 1.25 volumes of 100% Ethanol was added, mixed by pippetting, and added to the supplied column. The column was washed as recommended and the RNA was eluted with 2 x 50 ul of the kit supplied elution buffer heated to 98 °C. Total RNA was sent to CoFactor Genomics (St. Louis, MO) for construction of the cDNA libraries, and sequencing using one lane of a Solexa Illumina flow plate. Reactions were single end, 30 bp reads. Contigs were assembled and aligned to the *P. triticina* genome using Cofactor proprietary software.

Sequence analysis and database searches

Haustorial EST's were aligned to *P. triticina* whole genome using BLAST (Altschul *et al*., 1990) to eliminate plant contamination. Assembled contigs and unigenes were analyzed for function and homologies using BLASTn and BLASTx. Comparisons were made to the nr database at NCBI with default settings. BLAST2GO (http://www.blast2go.org/; Conesa *et al.,* 2005) was used for functional annotation of the EST's with the settings of QBLAST-NCBI low complexity filter, annotation cutoff of 55, and GO weight of 5. Alignments back to the *P. triticina* genome used the *Puccinia* group database (http://www.broadinstitute.org/annotation/genome/puccinia_group/Blast.html) using the settings for BLAST alignment were BLOSUM62, FILTER (YES), alignment type = gapped, and threshold of e -3. Sequences were screened for repetitive elements using CENSOR and default

settings (Kohany *et al.,* 2006). Open reading frames were identified with FGENESH (http://linux1.softberry.com/berry.phtml). Identified ORF, were analyzed for the presence of a predicted nuclear localization signal using PredictNLS online (http://cubic.bioc.columbia.edu/cgi/var/nair/resonline.pl; Cokol *et al*., 2000) with default settings, and also scanned for motifs using MEME Suite of motif-based sequence analysis tools. Settings for the searches were: optimum number of sites (more than 2 and less than 100), occurrence of a single motif distributed among the sequences as (any number of sequences), and maximum of 6 motif per sequence (*www.meme.sdsc.edu* ; Timothy *et al*, 2006). Secondary structure comparison was done with LOPP@BioHPC (http://cbsuapps.tc.cornell.edu/loopp.aspx) with default settings. Predicted secreted proteins were identified with the program SignalP v. 3.0 (http://www.cbs.dtu.dk/services/SignalP/; Bendtsen *et al*., 2004) with the settings: Organism group: Eukaryotes; output format: standard; Methods: Neural Network (NN) and Hidden Markov models (HMM); graphics: GIF (inline); output format: standard. Score values from HMM and NN ≥0.6, where selected as positive for secretion signal.

Semiquantitative RT-PCR

To evaluate the expression of selected candidate genes during infection, total RNA was isolated from uninfected seedling wheat leaves at the 2-3 leaf stage, infected wheat leaves harvested 1, 2, 3, 4, 5 and 6 dpi; *in vitro* germinated urediniospores at 30 min, 1, 2, and 3 h, and isolated haustorium. cDNA was made using First-Strand cDNA Synthesis SuperScript II RT (Invitrogen, California) following the manufacturer's instruction. Primers were designed for the coding region of the fungal proteins and included a *Bam*HI cloning site (Appendix A). Forward primers included the start ATG codon and the following19 bases. Reverse primers included the

last 19 bases of the coding region and the termination codon. Expression control primers were designed for the *P. triticina* ß-tubulin (PTTG_00759.1) and to validate cDNA quality and PCR reaction success. The amplicons were visualized on a 1 % agarose gel in 1X TAE buffer.

Results

Sequence analysis from haustorium specific EST's

From the leaf rust strain PBJL, haustorium specific ESTs were sequenced and from 4,128 clones, 6,493 cDNA sequences were obtained and assembled using CAP3 (Huang and Madam, 1999) into 260 contigs and 2,612 singlets for a total of 2,878 unigenes. Comparison of the unigenes using BLASTn whole-genome shotgun reads (wgs) identified 188 non-redundant *P. triticina* specific sequences (118 contigs and 70 singlets) and discriminated 2,690 sequence as empty vectors or because homology with other organisms sequences. BLASTX was performed and 96 sequences had no similarities with sequences in the public database and 24 had significant similarities ($E \geq 10^{-5}$) to proteins from non-fungal organisms. From the remaining sequences, 68 sequences were fungal specific, 15 aligned to proteins from ascomycetes and 53 aligned with basidiomycetes. The majority of the sequences (41 sequences) were associated with predicted proteins with unknown function in the basidiomycetes, such as; *Ustilago maydis, Cryptococcus neoformans, Laccaria bicolor, Schizophyllum commune*, *Postia placenta, Malassezia globosa, Coprinopsis cinerea,* and ascomycetes such as; *Penicillium chrysogenum , Yarrowia lipolytica, Scheffersomyces stipitis, Botrynia fuckeliana. Giberella zea* and *Neurospora crassa* (Table 2). Similarities with proteins related to metabolic processes and

energy production were the second largest group. Within this group two proteins were of specific interest. One had similarity to a haustorium expressed protein from *Melampsora medusae* (2.0E-37). Another had similarity with a SNARE YKT6 protein from *Laccaria bicolor* (1.0E-63) which is involved in membrane fusion events and a secretory-pathway in fungi (Kienle *et al*., 2008).

Further analysis for provisional annotation was done with BLAST2GO, which identified potential functions for 106 sequences (56%=106/188). This analysis identified 77/106 of the ESTs to be of fungal origin, while 29 ESTs were found to be similar to proteins from plants, animals or different organisms. The majority (26) of the EST's were associated with hypothetical proteins and cellular component proteins. The second major (14) group was associated with protein binding and oxidoreductase functions (Figure 2). Twelve unigenes were associated with ribosomal proteins and metabolic functions such as nucleic acid binding, catalytic activity, transferase, transaminase, isomerase and lyase activity. Several proteins involved in fungal development were identified: two were associated with spore germination; two were associated with mycelium development; two proteins are membrane associated; and two proteins were associated with senescence. There were four unigenes for heat shock proteins and two involved in oxidative stress. Associations were found for cutinase (1) and cell wall glucanase (1) (Figure 2).

Sequence analysis from EST infected tissue

Since isolation of haustoria is difficult and yield is low, cDNA libraries were made from heavily infected tissue. Illumina sequencing was used and the sequence was assembled back to the genome and 650 EST unigenes were identified. Comparison using BLASTn whole-genome

shotgun reads (wgs), discriminated 149 sequences with no hits into the public database and 501 sequences with hits. From 501 sequences, 205 aligned specifically with *P. triticina* sequences, 39 with *P. graminis* sequences and 257 had similarity with sequences from different organisms. BLASTX alignments to the NCBI non-redundant database showed that 66 of the 244 *Puccinia*-specific sequences had no similarity to proteins of known function. Of the 178 ESTs remaining, 100 had homologies to proteins from basidiomycetes and 34 from ascomycetes (Table 3). Fungal sequences were associated mostly with hypothetical proteins from *Ustilago maydis, Uromyces viciae fabae, Coprinopsis cinerea, Malassezia globosa, Laccaria bicolor, Postia placenta, Schizophyllum commune*, *Allomyces macrogynus, Moniliophthora perniciosa Cryptococcus neoformans, Talaromyces stipitatus, Scheffersomyces stipitis, Tuber melanosporum, Aspergillus terreus, Nectria haematococca, Podospera anserine, Sordaria macrospora, Phaeosphaeria nodorum, Trichophyton verrucosum, Aspergillus clavstus, Candida albicans, Sordaria macospora, Penicillium chrysogenum, Neurospora crassa, Paracoccidioides brasiliensis* and *Venturia inaequalis*. Relevant matches included a chitinase from *P. triticina* ($4E^{-76}$), NMT1, a plant induced protein from *Uromyces viciae-fabae* ($1.0E^{-36}$; Hahn and Mendgen, 1997), and FK506 binding protein (involve in protein trafficking and folding) from *Malassezia pachidermatis* ($1.00E^{-15}$;). Two ESTs had similarity with an argonaute like protein from *Laccaria bicolor* ($1.00E^{-31}$) and *Schizoaccharomyces pombe* ($1.00E^{-19}$), respectively. Also, similarities with heat shock protein 90 from *Schizophyllum commune* ($9E^{-15}$) was identified. BLAST2GO analysis indicated that 71 of the 244 *Puccinia* specific sequences were without alignments. Similarities at the $E \geq 10^{-6}$ level of significance were found for 173 sequences, of which 26 were associated with plant proteins. From 147 fungal specific sequences, the largest group (26%= 39/147) was associated with predicted proteins with unknown function. The second

largest groups had similarities with ribosomal proteins (16), or a peptide binding function (14). Interesting hits revealed function in response to stress, such as heat shock protein 90 and potential role in the infection for the chitinase from *P. triticina* (Figure 3).

Bioinformatic strategy for candidate effector identification

Puccinia specific sequences were subject to FGENESH to identify complete open reading frames (ORF) and predict translation products. One of the few distinguishing features among effectors of filamentous fungi and oomycetes is the presence of a signal peptide (SP) for secretion. Identification of putative secreted proteins by SignalP 3.0 involves HMM and NN algorithms. Ideally, both scores should have a value of 1.00 for high confidence in the prediction. From ESTs derived from haustoria specific sequences (Table 5) and infected tissue, 16 predicted proteins were identified. Gene presence was validated by genomic PCR and transcription was verified by RT-PCR with cDNA from germinated spores and infected plants (6 dpi). Non-inoculated plants were used as a negative control. Three sequences, Pt59, Pt65 and Pt67, did not amplify from genomic DNA and were eliminated from further analysis. Pt74 contained a transposon domain and was also removed. The 10 remaining candidates were considered avirulence candidates and evaluated further (Table 6). All were found in the genomic sequence of race BBBD and none contained a nuclear localization signal nor were they considered repetitive elements. The number of cysteine residues ranged from 0-12, and the SP length ranged from 18-24 amino acids.

The majority of cloned Avr genes from filamentous plant pathogens are small-secreted proteins and the presence of an N-terminus signal peptide (SP) allows for secretion of the protein in the interface between the pathogen and the host. The prediction of SP relies in the presence of

charged, hydrophobic and polar regions between the N-terminus and the signal peptide cleavage site (Lee *et al*., 2003). To compare the secreted nature of Pt candidates, a scatter diagram of predicted mean S and HHM scores from annotated filamentous fungi and oomycetes Avr, and Pt candidates were plotted (Figure 5). Selected *Puccinia* candidates clustered at the higher quadrant on the graph, along with the majority of known filamentous fungi and oomycetes Avr genes. The highest HMM/NN scores belong to AVR3a and AVRblb1, both *Phytophthora infestans* effectors, and the lowest scores were shown in AVRblb2 (also a *P. infestans* effector), all of them cytoplasmic effectors (Armstrong *et al*., 2005, Vleeshouwers *et al*.,2008; Oh *et al*., 2009). Also, apoplastic effectors have high score values such as AVR2 (0.959/1), AVR4 (0.909/1) and AVR9 (0.966/0.999) from *Cladosporium fulvum* (VanDen Ackerveken *et al*., 1993; Dixon *et al* 1996). BLAST2GO utilizes a sophisticated algorithm that considers similarity, extension of the homology, database, gene ontology (GO) hierarchy, and quality of the original annotations. When several GO terms are associated to a BLAST hit BLAST2GO uses the mean value of the probability of all alignments to derive a GO value (Conesa *et al*., 2005). Provisional functional annotation with BLAST2GO was performed for Pt candidates. Half of the candidates have no annotation (NA; Table 7). Candidate Pt58 has similarity with a cell wall glucanase and Pt63 homology to a hypothetical protein from the basidiomycete, *Schizophyllum commune*. Pt68 is similar to a superoxide dismutase from *T. aestivum* and candidate Pt69 is similar to a predicted protein from the ascomycete *Botryotinia fuckeliana* ($4.43E^{-07}$). Interestingly, Pt70 is homologous to hesp-379-like protein from the rusts *Melampsora medusae* f. sp. and *Melampsora lini*.

Candidate effector characterization

The *P. triticina* genome is estimated to be 100 to 120 Mb and the large genome size is attributed to abundant repetitive sequences. In order to elucidate the probability that the candidates were of repeated sequence origin, the candidates were analyzed by GiRi (*http://www.girinst.org/censor/index.php*). None were positive for fungal repetitive sequences. Avirulence candidates were then subjected to NLS screening http://cubic.bioc.columbia.edu/cgi/var/nair/resonline.pl. None of them have an obvious NLS motif. No similarity with known proteins was identified at the secondary structure level by LOPP@BioHPC. The majority of the reported Avr genes from filamentous microorganisms are small secreted proteins, and often they are cysteine-rich proteins (Stergiopoulos and de Wit 2009). In Pt candidates, four proteins have more than 3 cysteine residues; Pt3 (five), Pt51 (nine), Pt68 (five), and Pt72 (twelve) cysteine residues (Table 6). Recently, Godfrey *et al* (2010) identified a potential N-terminal motif, Y/F/WxC-motif, in barley powdery mildew effectors (*Blumeria graminis* f.sp. *hordei*). It is present in Pt51 at position 89, and Pt71 at position 163. In order to identify additional potential motifs shared by the novel Pt candidates and the cloned Avr proteins, all sequences were submitted to MEME (Multiple Em for Motif Elicitation). No apparent motifs were identified in Pt candidates.

Semiquantitative RT-PCR validation

Primers for each candidate sequence were tested in samples derived from wheat leaves (**p**), infected plants harvested six dpi (**i**) and *in vitro* germinated spores (**sp**) (Figure 6). Quality of the cDNA was evaluated using *P. triticina* beta tubulin primers. Amplification from both sources of cDNA, **sp** and **i**, was obtained from eight candidates Pt12, Pt27, Pt58, Pt63, Pt68,

Pt69, Pt70, and Pt71. Candidates Pt3 and Pr51 were only expressed in rust infected tissue (Figure 6), and time course expression was also evaluated from leaf rust infected leaves at 0, 1, 2, 3, 4, 5 and 6 dpi for these genes. Candidate genes that were expressed in both **sp** and **i** do not show any change in the pattern of expression from 1 to 6 dpi. Therefore, expression was evaluated at early stages of spore germination. Critical times for spore germination for were previously determined (Appendix B). Expression at 30 minutes, 1, 2 and 3 hours were evaluated by RT-PCR, with the conserved *Puccinia* beta-*tubulin* gene as quality control. No change in the level of expression was observed (Figure 6). For candidate genes, Pt3 and Pt51, which were expressed only in infected tissue, there was change in the gene expression over the time. A gradual increase over time course, from 1 to 6 days, was detected. An additional experiment was performed to corroborate specific expression in the leaf rust haustorium. Since the total RNA consists of a mixture from wheat chloroplast and isolated haustoria, mock inoculated plants were subject to haustorium isolation protocol as a negative control. Amplicons from both Pt3 and Pt51 were obtained only in haustorial samples (Figure 6).

Discussion

Wheat leaf rust is an important disease that is very aggressive in Kansas. Understanding the pathogen biology is necessary to develop new strategies of disease control. Classic pathology has shown how the fungus infects a plant, the urediniospore lands on the leaf surface, germinates, and the germ tube attempts to find a stomate. If successful, an appresorium will form and begin penetrating the stomata. It is the first few hours of infection that determine whether a compatible interaction will take place. The fungus will secrete a host of proteins that are intended to provide an environment within the plant for the fungus to complete its life cycle.

On the other hand, the plant is perceiving the infection and attempts to defend itself. Fungi have evolved a class of proteins that are called effectors that will block and skirt host defenses while transforming the cell to become a nutrient sink. Both monocot and dicot plants have developed a resistance gene system that recognizes specific effectors and ligands and will induce cell-death to prevent spread of the infection. By understanding what effectors are present in the early stages of infection or are present in important fungal structures, the dance between host and pathogen can be understood.

The ultimate goal of this research was the identification of leaf rust effector avirulence factors from a collection of expressed genes during the infection. This work is difficult because of the many challenges in doing basic genetic and biochemical research. *Puccinia triticina* is an obligate biotroph and cannot be cultured *in vitro*. An alternate host is required for sexual crosses, but it is not present in the wild in North America. So, avenues had to be explored to expose the factors involved in infection. ESTs have proved to be a useful tool to provide these answers. Previous work in flax rust (*M. lini*) identified 429 ESTs from haustoria. Twenty-one were secreted proteins and four of them co-segregated with avirulence loci in the fungus (Catanzarity *et al*., 2006). In this research, 432 EST's were derived from haustoria and infected plants. Fifteen secreted proteins were identified and 10 were selected as potential avirulence candidates. Function could be assigned to many of the expressed tags, but 162 ESTs had not predicted functions and represented a putative source of effectors.

A Summary of the distribution of *P. triticina* EST from haustorium-specific and infected tissue is showin in Table 4. Haustorium specific ESTs reveal sequences associated mostly with hypothetical proteins and proteins involved in metabolic process and biological energy

production. Similar findings were reported from analysis in other haustoria forming pathogens, such as *Blumeria graminis, Pucccinia striiformis* and *Uromyces fabae* (Godfrey *et al*., 2009; Yin *et al*., 2009; Hahn and Mendgen, 1997). Two *Puccinia triticina* (Pt) avirulence candidates, Pt3 and Pt51, were expressed only in the haustoria and are novel, small cysteine-rich secreted proteins. Avr genes encode small proteins with N-terminal signal peptides and are often cysteine-rich (Stergiopoulus and de Wit, 2009). Apparently, the small size and the secretion signal peptide facilitate the secretion from the pathogen, and the cysteine residues guarantee stability in the protein by forming disulfide bonds and prevent protease degradation (Stergiolopulos and de Wit, 2009). Eight of our candidates are expressed at an early stage of infection. Pt71 is a cysteine rich protein with 12 cys residues, similar to AvrP123 from *M. lini*, which has 11 cysteine residues (Catanzariti *et al*., 2006). Pt68 has 5 cysteine residues like AVRLm6 from *Leptosphaeria maculans,* which has 6 cysteine residues (Fudal *et al*. 2007). Although 6 candidates have less than 3 cysteine residues or none (as in the case of Pt70), this does not interfere with the criteria of selection, since some cloned effectors are also poor in cysteine, such as AVRL567 and AVRM from *M. lini* (Catanzariti *et al*., 2006), PWL2 from *M. oryzae* (Kang *et al*. 1995) and AVRLm1 from *L. maculans (*Gout *et al*. 2006), all of them with 1 cysteine residue.

The strategy for candidate characterization is illustrated in Figure 4. All the selected candidates fulfill the criteria of being small-secreted proteins. None of them have a repetitive origin, so they are not retrotransposon sequences; neither do they have nuclear localization signal, so their function does not involve translocation to the host nuclei. And they have no significant similarities in secondary structures with proteins in the data base. A conserved motif would be beneficial for efficient identification of effectors; however, there is no clear indication

about conserved motifs. Candidates Pt 51 and Pt71 have the Y/F/WxC motif found in *Blumeria graminis* (Godfrey *et al.,* 2010), but it is not known if it is effective or just chance.

Candidate Pt58 has similarity with a cell wall glucanase and the corresponding locus, *P. triticina* (PTTG_00152.1), encodes a protein domain similar to glycosyl hydrolase family 16, which indicates a potential role in disrupting plant cell wall and facilitate the infection. It is also possible that after secretion, the plant might detect this effector and start a PAMP triggered immunity (PTI) response (Jones and Dangl, 2006). Pt70 has a corresponding locus (PTTG_05971.1) with a protein domain that is predicted to be a developmentally regulated MAPK interacting protein. Such domain belongs to a protein that appear to be involve in fruiting body formation and in host attack in *Lentinula edodes* (basidiomycete; Szeto *et al*., 2007) which at the same time, share close similarity with HESP-379, a secreted protein expressed in haustoria in *M. lini* (Szeto *et al*., 2007). Thus, it is possible that Pt70 has an active role during leaf rust infection.

Conclusion

Ten predicted secreted proteins specific to *Puccinia triticina* were identified from a cDNA library of expressed sequences during infection. As demonstrated in *P. striiformis* and *M. lini* (Yin *et al*., 2009; Catanzarity *et al*., 2006), generation of an EST collection is an important strategy that can be used to gain understanding about genes expressed during infection and therefore enable the understanding of the pathosystem molecular interaction. Although genes expressed in haustoria are mostly related with metabolic processes, evaluation of secreted proteins will enable Avr gene identification. So far, the paradigm about fungal effectors is that they are novel proteins. This research is the first study reported about secreted proteins expressed

during wheat leaf rust infection, and it may lead for a further research to validate the candidates as secreted proteins with avirulence function.

Table 2. *P. triticina* haustorium specific ESTs annotations based on BLASTX algorithm search.

Gene ID	Alignment	Score (bits)	E value*
Basidiomycetes			
Contig154	sp|P50138|ACT_PUCGR RecName: Full=Actin >CAA54848 *Puccinia graminis*	306	3.00E-81
PT0333.A12.S6Wu.ptih|	sp|P50138|ACT_PUCGR RecName: Full=Actin >|CAA54848*Puccinia graminis*	409	1.0E-112
Contig129	ref|XP_757799.1| hypothetical protein UM01652.1 [*Ustilago maydis*	305	8.0E-81
Contig33	ref|XP_758608.1| hypothetical protein UM02461.1 [*Ustilago maydis*	223	7.0E-57
Contig226	ref|XP_758664.1| guanine nucleotide-binding protein subunit *Ustilago maydis*	218	9.0E-55
Contig240	ref|XP_762540.1| hypothetical protein UM06393.1 [*Ustilago maydis*	180	2.0E-43
Contig231	ref|XP_756882.1| hypothetical protein UM00735.1 [*Ustilago maydis*	171	1.0E-40
PT03336.F03.C21.ptih|	ref|XP_761326.1| hypothetical protein UM05179.1 [*Ustilago maydis*	107	2.0E-27
Contig35	ref|XP_757887.1| hypothetical protein UM01740.1 [*Ustilago maydis*	92	6.0E-17
PT03321.F01.S6Wu.ptih|	ref|XP_759346.1| hypothetical protein UM03199.1 [*Ustilago maydis*	395	1.0E-108
Contig26	emb|CAH10835.1| major alcohol dehydrogenase [*Uromyces viciae-fabae*]	214	3.00E-54
Contig29	gb|ABS86270.1| hesp-379-like protein [*Melampsora medusae f. sp*.	160	2.00E-37
PT03339.F02.S6Wu.ptih|	ref|XP_571604.1| hypothetical protein CNF03180 [*Cryptococcus neoformans*	164	1.00E-38
Contig144	ref|XP_571820.1| pria protein precursor [*Cryptococcus neoformans*	87	2.00E-15
Contig12	ref|XP_572805.1| hypothetical protein [*Cryptococcus neoformans*	135	4.00E-30
Contig157	ref|XP_775732.1| hypothetical protein CNBD4610 [*Cryptococcus neoformans*	68	2.00E-11
Contig150	ref|XP_569119.1| intracellular protein transport-rel.prot.*Cryptococcus neoformans*	296	2.00E-78
Contig162	ref|XP_570152.1| NADH-ubiquinone oxidored.51kDa subunit [*C. neoformans*	202	3.00E-50
Contig96	ref|XP_571714.1| electron transporter, transferring electrons [*C. neoformans*	412	1.0E-113
PT03334.C04.S6Wu.ptih|	ref|XP_001873339.1| predicted protein [*Laccaria bicolor* S238N-H8	64	8.00E-09
Contig228	ref|XP_001886114.1| anthranilate phosphoribosyltransferase, [*Laccaria bicolor*	162	5.00E-38
Contig190	ref|XP_001873508.1| predicted protein [*Laccaria bicolor* S238N-H8	73	1.00E-11
Contig236	ref|XP_001873779.1| predicted protein [*Laccaria bicolor* S238N-H8	76	3.00E-12
Contig13	ref|XP_001874166.1| predicted protein [*Laccaria bicolor* S238N-H8	255	2.00E-66
Contig206	ref|XP_001876506.1| predicted protein [*Laccaria bicolor* S238N-H8	60	7.00E-08
PT03316.C03.C21.ptih|	ref|XP_001880628.1| predicted protein [*Laccaria bicolor* S238N-H8	192	3.00E-47
Contig193	ref|XP_001883636.1| predicted protein [*Laccaria bicolor* S238N-H8	213	3.00E-53

Gene ID	Alignment	Score (bits)	E value*
Contig238	ref|XP_001874606.1| predicted protein [*Laccaria bicolor* S238N-H8	692	0
PT0338.F10.C21.ptih|	ref|XP_001889487.1| SNARE protein YKT6 [*Laccaria bicolor* S238N-H	246	1.00E-63
Contig131	ref|XP_002474120.1| hypothetical protein POSPLDRAFT_95351 [*Postia placenta*	206	4.00E-51
PT03328.E01.S6Wu.ptih|	ref|XP_002475227.1| 60S ribosomal protein L32 [*Postia placenta*	193	6.00E-48
PT03322.B01.C21.ptih|	ref|XP_002471524.1| predicted protein [*Postia placenta* Mad-698-R	118	6.00E-25
Contig255	ref|XP_003035960.1| hypothetical protein SCHCODRAFT_ [*Schizophyllum commune*	317	1.00E-84
PT0335.A05.S6Wu.ptih|	ref|XP_003035960.1| hypothetical protein SCHCODRAFT_ [*Schizophyllum commune*	450	1.0E-124
Contig181	ref|XP_003035279.1| glycosyltransferase family 50 protein [*Schizophyllum commune*	136	2.00E-30
Contig257	ref|XP_003027035.1| hypothetical protein SCHCODRAFT_ [*Schizophyllum commune*	81	4.00E-14
Contig146	ref|XP_003031384.1| hypothetical protein SCHCODRAFT_ [*Schizophyllum commune*	59	1.00E-06
PT0332.A04.S6Wu.ptih|	ref|XP_001728955.1| hypothetical protein MGL_3949 [*Malassezia globosa*	65	2.00E-09
Contig139	ref|XP_001732063.1| hypothetical protein MGL_0656 [*Malassezia globosa*	75	4.00E-12
Contig227	ref|XP_001828941.1| hypothetical protein CC1G_03735 [*Coprinopsis cinerea*	77	6.00E-13
PT03333.C10.C21.ptih|	ref|XP_001829157.1| hypothetical protein CC1G_01837 [*Coprinopsis cinerea*	75	2.00E-12
Contig18	ref|XP_001836593.2| hypothetical protein CC1G_06180 [*Coprinopsis cinerea*	157	1.00E-36
PT03322.B06.S6Wu.ptih|	ref|XP_001836950.2| acyl-CoA dehydrogenase [*Coprinopsis cinerea*	198	5.00E-49
Contig73	ref|XP_001839344.1| 60S ribosomal protein L18-B [*Coprinopsis cinerea*	138	6.00E-46
Contig248	ref|XP_002911600.1| hypothetical protein CC1G_14133 [*Coprinopsis cinerea*	189	7.00E-46
Contig188	ref|XP_002911655.1| NADPH oxidase regulator NoxR [*Coprinopsis cinerea*	62	3.00E-08
Contig68	ref|XP_001828607.1| hypothetical protein CC1G_10278 [*Coprinopsis cinerea*	90	2.00E-16
PT03330.A05.S6Wu.ptih|	ref|XP_001828739.2| 60s ribosomal protein l15 [*Coprinopsis cinerea*	165	2.00E-39
Contig229	ref|XP_002910614.1| phosphopantothenoylcysteine decarboxylase[*Coprinopsis cinerea*	168	4.00E-49
Contig106	dbj|BAJ04691.1| glyceraldehyde-3-phosphate dehydrogenase [*Helicobasidium mompa*	536	1.0E-150
Contig210	gb|ABY85444.1| homing endonuclease [*Agaricus bisporus*]	257	2.00E-66
Contig93	ref|YP_003795686.1| NADH dehydrogenase subunit 3 [*Phakopsora meibomiae*	102	2.00E-20
Contig165	sp|Q01200|PRIA_LENED RecName: Full=Protein priA; Flags: *Lentinula edodes*	76	6.00E-12

Gene ID	Alignment	Score (bits)	E value*
Ascomycetes			
Contig108	ref|XP_001383614.1| hypothetical protein PICST_57317 [*Scheffersomyces stipitis*	124	2.00E-35
PT03325.F04.C21.ptih|	ref|XP_001383614.1| hypothetical protein PICST_57317 [*Scheffersomyces stipitis*	120	4.00E-30
PT03341.B08.S6Wu.ptih|	ref|XP_505403.1| YALI0F14223p [*Yarrowia lipolytica*] >gi|49651273	253	2.00E-86
Contig169	ref|XP_002493706.1| Vacuolar transporter chaperone (VTC) involved. *Pichia pastoris*	187	2.00E-45
PT03327.F03.C21.ptih|	ref|XP_447316.1| hypothetical protein [*Candida glabrata* CBS 138]	60	1.00E-07
Contig116	ref|XP_001222375.1| hypothetical protein CHGG_06280 [*Chaetomium globosum*	80	2.00E-13
PT03338.H04.S6Wu.ptih|	ref|XP_001222401.1| hypothetical protein CHGG_06306 [*Chaetomium globosum*	75	7.00E-12
Contig235	ref|XP_001229743.1| hypothetical protein CHGG_03227 [*Chaetomium globosum*	137	4.00E-31
Contig171	ref|XP_001538934.1| hypothetical protein HCAG_06539 [*Alejomyces capsulatus*	259	3.00E-67
Contig7	ref|XP_001553092.1| predicted protein [*Botryotinia fuckeliana* B0...	61	4.00E-07
Contig21	ref|XP_002486307.1| transcription elongation factor SPT6, putati. [*Talaromyces stipitatus*	250	3.00E-64
Contig186	ref|XP_002559320.1| Pc13g08950 [*Penicillium chrysogenum*	78	4.00E-13
Contig213	ref|XP_003066515.1| Decaprenyl-diphosphate synthase , putative [*Coccidioides posadasii*	128	6.00E-28
Contig225	ref|XP_382273.1| hypothetical protein FG02097.1 [*Gibberella zeae*	82	4.00E-14
Contig244	ref|XP_961100.1| hypothetical protein NCU03753 [*Neurospora crass*	59	2.00E-07
Other organisms			
PT0332.D07.C21.ptih|	ref|ZP_03066015.1| IS2 transposase orfB [*Shigella dysenteriae*	349	1.00E-94
Contig43	ref|ZP_02244999.1| two-component system sensor protein [*Xanthomonas oryzae pv. oryzicola*	111	2.00E-22
Contig109	ref|YP_003322077.1| 2-oxoglutarate dehydrogenase, E1 subunit *[Thermobaculum terrenum*	67	1.00E-09
Contig175	ref|ZP_03294132.1| hypothetical protein CLOHIR_02084 [*Clostridium hiranonis*	60	8.00E-07
Contig2	gb|ACO90195.1| superoxide dismutase [*Triticum aestivum*]	203	2.00E-50
Contig195	ref|XP_002488947.1| hypothetical protein SORBIDRAFT_ *Sorghum bicolor*	158	7.00E-37
Contig176	gb|ACR38454.1| unknown [*Zea mays*]	125	2.00E-27
Contig167	ref|XP_002488959.1| hypothetical protein SORBIDRAFT_ [*Sorghum bicolor*	101	3.00E-23
PT03336.C06.C21.ptih|	emb|CAB72466.1| putative protein [*Arabidopsis thaliana*]	108	4.00E-22
PT03326.E10.S6Wu.ptih|	gb|ACR38454.1| unknown [*Zea mays*]	105	3.00E-21

Gene ID	Alignment	Score (bits)	E value*
Other organisms			
PT03325.C11.S6Wu.ptih|	gb|ACR38454.1| unknown [*Zea mays*]	103	1.00E-20
PT03316.C12.S6Wu.ptih|	dbj|BAB33421.1| putative senescence-associated protein [*Pisum sativum*	60	1.00E-13
PT03336.B08.S6Wu.ptih|	ref|XP_001698950.1| hypothetical protein CHLREDRAFT_155068	72	4.00E-11
PT03319.D09.C21.ptih|	emb|CBJ34259.1| expressed unknown protein [*Ectocarpus siliculosus*]	70	7.00E-11
PT03338.G09.S6Wu.ptih|	gb|ACR38454.1| unknown [*Zea mays*]	60	9.00E-08
Contig198	gb|AAX95493.1| Retrotransposon gag protein, putative [*Oryza sativa*	55	9.00E-06
PT03323.D06.S6Wu.ptih|	ref|XP_001651807.1| cystinosin [*Aedes aegypti*] >gi|108878114|gb|	132	1.00E-29
Contig199	gb|AAX30301.1| unknown [*Schistosoma japonicum*]	110	5.00E-23
Contig41	ref|XP_001618200.1| hypothetical protein NEMVEDRAFT_ [*Nematostella vectensis*	110	8.00E-23
Contig60	ref|XP_001895031.1| hypothetical protein Bm1_17870 [*Brugia malay*	95	3.00E-18
Contig230	ref|XP_001895031.1| hypothetical protein Bm1_17870 [*Brugia malay*	86	6.00E-15
Contig6	ref|XP_598451.2| PREDICTED: proteasome activator subunit 3-like [*Bos taurus*	73	3.00E-11
PT0335.H02.C21.ptih|	ref|XP_001625237.1| predicted protein [*Nematostella vectensis*]	63	4.00E-08
Contig258	ref|XP_002824448.1| PREDICTED: hypothetical protein [*Chlamydomonas reinhardtii*	57	8.00E-07

*E values recorded at $E>10^{-5}$ confidence level.

Table 3. *P. triticina* infected tissue ESTs annotations, based on BLASTX algorithm.

Gene ID	Alignment	Score (bits)	E value*
Basidiomycetes			
family_284_mult_7	gb|AAP42830.1| alcohol dehydrogenase [*Puccinia triticina*]	329	1.00E-88
family_547_mult_13	sp|P50138|ACT_PUCGR RecName: Full=Actin >gi|460993|emb|CAA54848 *Puccinia graminis*	287	4.00E-76
family_616_mult_9	gb|AAP42832.1| chitinase [*Puccinia triticina*]	246	7.00E-64
family_425_mult_12	sp|P50138|ACT_PUCGR RecName: Full=Actin >gi|460993|emb|CAA54848 *Puccinia graminis*	235	2.00E-60
family_513_mult_11	gb|AAP42830.1| alcohol dehydrogenase [*Puccinia triticina*]	205	2.00E-51
family_545_mult_10	gb|AAP42832.1| chitinase [*Puccinia triticina*]	201	3.00E-50
family_643_mult_7	gb|AAP42833.1| putative sorbitol-utilization protein [*Puccinia triticina*	130	6.00E-29
family_296_mult_20	ref|XP_756613.1| hypothetical protein UM00466.1 [*Ustilago maydis*	70	3.00E-10
family_94_mult_12	ref|XP_761118.1| hypothetical protein UM04971.1 [*Ustilago maydis*	210	4.00E-53
family_429_mult_6	ref|XP_758499.1| hypothetical protein UM02352.1 [*Ustilago maydis*	203	7.00E-51
family_637_mult_8	ref|XP_759446.1| hypothetical protein UM03299.1 [*Ustilago maydis*	176	9.00E-43
family_335_mult_10	ref|XP_761118.1| hypothetical protein UM04971.1 [*Ustilago maydis*	144	3.00E-33
family_522_mult_7	ref|XP_762483.1| hypothetical protein UM06336.1 [*Ustilago maydis*	142	2.00E-32
family_568_mult_23	ref|XP_762595.1| hypothetical protein UM06448.1 [*Ustilago maydis*	122	2.00E-26
family_444_mult_16	ref|XP_758408.1| hypothetical protein UM02261.1 [*Ustilago maydis*	120	1.00E-25
family_580_mult_10	ref|XP_761066.1| hypothetical protein UM04919.1 [*Ustilago maydis*	116	1.00E-24
family_644_mult_6	ref|XP_761237.1| hypothetical protein UM05090.1 [*Ustilago maydis*	110	6.00E-23
family_536_mult_8	ref|XP_756944.1| hypothetical protein UM00797.1 [*Ustilago maydis*	105	1.00E-21
family_573_mult_11	ref|XP_756731.1| hypothetical protein UM00584.1 [*Ustilago maydis*	428	1.0E-118
family_259_mult_56	sp|O00057|NMT1_UROFA RecName: Full=Protein NMT1 homolog; *Uromyces viciae-fabae*	657	0
family_319_mult_28	sp|O00061|CP67_UROFA RecName: Full=Cytochrome P450 67; *Uromyces viciae-fabae*	810	0
family_592_mult_13	sp|O00058|MTDH_UROFA RecName: Full= Probable NADP-dependent mannitol dehydrogenase	294	2.00E-78
family_391_mult_10	sp|O00057|NMT1_UROFA RecName: Full=Protein NMT1 homolog; *Uromyces viciae-fabae*	153	6.00E-36
family_207_mult_38	sp|Q9UVF8|THI4_UROFA RecName: Full=Thiazole biosynthetic enzyme, *Uromyces viciae-fabae*	616	1.0E -174

Gene ID	Alignment	Score (bits)	E value*
	e		
family_88_mult_17	emb|CAH10835.1| major alcohol dehydrogenase [*Uromyces viciae-faba*	618	1.0E -175
family_561_mult_9	gb|ABS86197.1| 14-3-3 protein [*Melampsora laricis-populina*	269	8.00E-71
family_524_mult_14	gb|ACR44285.1| NADH dehydrogenase subunit 6 [*Melampsora aecidioides*	197	5.00E-49
family_157_mult_15	gb|ABS86591.1| thioredoxin [*Melampsora medusae f. sp. deltoidis*	102	2.00E-20
family_597_mult_8	ref|XP_568352.1| galactose metabolism-related protein [*Cryptococcus neoforma*	92	2.00E-17
family_593_mult_8	ref|XP_566460.1| 60s ribosomal protein l9 [*Cryptococcus neoforma*	176	7.00E-43
family_431_mult_16	ref|XP_567910.1| transaldolase [*Cryptococcus neoforma*	255	3.00E-66
family_388_mult_12	ref|XP_567211.1| hypothetical protein [*Cryptococcus neoforma*	75	3.00E-12
family_147_mult_13	ref|XP_568302.1| ATP:ADP antiporter [*Cryptococcus neoforma*	295	2.00E-78
family_494_mult_9	ref|XP_001829437.1| enolase [*Coprinopsis cinerea*	252	1.00E-65
family_477_mult_10	ref|XP_001832234.1| hypothetical protein CC1G_02496 [*Coprinopsis cinerea*	224	2.00E-57
family_406_mult_18	ref|XP_001830342.2| transglycosylase SLT domain-containing protein [*Coprinopsis cinerea*	213	5.00E-53
family_603_mult_7	ref|XP_001830143.1| ribosomal protein S2 [*Coprinopsis cinerea*	204	2.00E-51
family_623_mult_8	ref|XP_001836588.2| transketolase [*Coprinopsis cinerea*	171	2.00E-41
family_540_mult_10	ref|XP_002911694.1| 40s ribosomal protein [*Coprinopsis cinerea*	169	1.00E-40
family_569_mult_7	ref|XP_001830449.1| ribosomal protein L12 [*Coprinopsis cinerea*	156	9.00E-37
family_551_mult_7	ref|XP_001837081.1| hypothetical protein CC1G_00217 [*Coprinopsis cinerea*	110	8.00E-23
family_631_mult_9	ref|XP_001828686.2| hypothetical protein CC1G_12661 [*Coprinopsis cinerea*	95	3.00E-18
family_566_mult_11	ref|XP_001837796.2| hypothetical protein CC1G_11441 [*Coprinopsis cinerea*	86	2.00E-15
family_353_mult_11	ref|XP_001828686.2| hypothetical protein CC1G_12661 [*Coprinopsis cinerea*	74	2.00E-11
family_647_mult_7	ref|XP_001828956.1| wos2 [*Coprinopsis cinerea*	58	5.00E-07
family_509_mult_12	dbj|BAD01553.1| FK506 binding protein [*Malassezia pachydermatis*]	86	1.00E-15
family_11_mult_10507	ref|XP_001728955.1| hypothetical protein MGL_3949 [*Malassezia globosa*	67	2.00E-09
family_214_mult_23	ref|XP_001728955.1| hypothetical protein MGL_3949 [*Malassezia globosa*	58	5.00E-07
family_45_mult_29	ref|XP_001728955.1| hypothetical protein MGL_3949 [*Malassezia globosa*	57	6.00E-07
family_584_mult_12	ref|XP_001873416.1| predicted protein [*Laccaria bicolor* S238N-H8	393	1.0E-107
family_423_mult_22	ref|XP_001878783.1| thaumatin-like protein [*Laccaria bicolor* S238N-H8	326	3.00E-87
family_144_mult_11	ref|XP_001874653.1| predicted protein [*Laccaria bicolor* S238N-H8	257	4.00E-67
family_275_mult_21	ref|XP_001878805.1| predicted protein [*Laccaria bicolor* S238N-H8	219	6.00E-55
family_608_mult_13	ref|XP_001876873.1| predicted protein [*Laccaria bicolor* S238N-H8	193	1.00E-47
family_563_mult_10	ref|XP_001884445.1| predicted protein [*Laccaria bicolor* S238N-H8	157	6.00E-37

Gene ID	Alignment	Score (bits)	E value*
family_483_mult_16	ref|XP_001876710.1| argonaute-like protein [*Laccaria bicolor* S238N-H8	139	1.00E-31
family_555_mult_13	ref|XP_001889571.1| carbohydrate esterase family 8 protein [*Laccaria bicolor* S238N-H8	134	1.00E-29
family_374_mult_16	ref|XP_001875220.1| proline-rich protein [*Laccaria bicolor* S238N-H8	90	1.00E-16
family_30_mult_66	ref|YP_003795384.1| COX1 *[Phakopsora pachyrhizi*] >gi|251765325|g	740	0
family_230_mult_41	ref|YP_003795698.1| NADH dehydrogenase subunit 5 [*Phakopsora meibomiae*	1052	0
family_280_mult_19	ref|YP_003795386.1| NAD1 [*[Phakopsora pachyrhizi*] >gi|251765327|g	355	3.00E-96
family_338_mult_38	ref|YP_003795690.1| cytochrome c oxidase subunit 3 [*Phakopsora meibomiae*	224	3.00E-57
family_122_mult_76	ref|YP_003795384.1| COX1 [*[Phakopsora pachyrhizi*] >gi|251765325|g...	199	1.00E-49
family_436_mult_34	ref|YP_003795690.1| cytochrome c oxidase subunit 3 [*Phakopsora meibomiae*	167	9.00E-40
family_268_mult_22	ref|YP_003795696.1| NADH dehydrogenase subunit 1 [*Phakopsora meibomiae*	132	2.00E-29
family_617_mult_13	ref|YP_003795685.1| NADH dehydrogenase subunit 2 [*Phakopsora meibomiae*	116	1.00E-24
family_113_mult_71	ref|YP_003795377.1| ATP8 [*[Phakopsora pachyrhizi*] >gi|251765318|	90	1.00E-16
family_312_mult_12	ref|YP_003795685.1| NADH dehydrogenase subunit 2 [*Phakopsora meibomiae*	78	3.00E-13
family_149_mult_170	ref|YP_003795374.1| ATP9 [*[Phakopsora pachyrhizi*] >gi|301353463|	75	3.00E-11
family_645_mult_7	ref|YP_003795693.1| ribosomal protein S3 [*Phakopsora meibomiae*]	54	9.00E-06
family_197_mult_67	ref|YP_003795688.1| ATP synthase subunit 6 [*Phakopsora meibomiae*	415	1.0E -114
family_496_mult_33	ref|YP_003795379.1| COX2 [*[Phakopsora pachyrhizi*] >gi|251765320|g...	430	1.0E -118
family_389_mult_27	ref|YP_003795691.1| NADH dehydrogenase subunit 4 [*Phakopsora meibomiae*	538	1.0E -151
family_137_mult_71	ref|YP_003795692.1| apocytochrome B [*Phakopsora meibomiae*] >gi|2...	634	1.0E -180
family_492_mult_24	ref|XP_002473551.1| predicted protein [*Postia placenta* Mad-698-R	149	6.00E-34
family_506_mult_8	ref|XP_002475553.1| 40S ribosomal protein S23 [*Postia placenta* Mad-698-R	127	4.00E-28
family_428_mult_16	ref|XP_002471524.1| predicted protein [*Postia placenta* Mad-698-R	118	3.00E-25
family_306_mult_15	ref|XP_002475683.1| hypothetical beta-fg [*Postia placenta* Mad-698-R	99	4.00E-19
family_614_mult_7	ref|XP_002472335.1| S-phase kinase-associated protein 1A-like protein [*Postia placenta*	157	3.00E-37
family_368_mult_10	ref|XP_003028981.1| hypothetical protein SCHCODRAFT_70041 [*Schizophyllum commune*	357	6.00E-97
family_635_mult_9	ref|XP_003034877.1| hypothetical protein SCHCODRAFT_65379 [*Schizophyllum commune*	224	2.00E-57
family_447_mult_17	ref|XP_003029135.1| hypothetical protein SCHCODRAFT_78835 [*Schizophyllum commune*	216	2.00E-54
family_541_mult_14	ref|XP_003034629.1| hypothetical protein SCHCODRAFT_84866 [*Schizophyllum commune*	197	1.00E-48
family_357_mult_17	ref|XP_003031830.1| hypothetical protein SCHCODRAFT_109135 [*Schizophyllum commune*	179	5.00E-43
family_473_mult_10	ref|XP_003038484.1| 40S ribosomal protein S27 [*Schizophyllum commune*	165	2.00E-39

Gene ID	Alignment	Score (bits)	E value*
family_326_mult_12	ref|XP_003028082.1| hypothetical protein SCHCODRAFT_85988 [*Schizophyllum commune*	156	7.00E-37
family_364_mult_10	ref|XP_003029367.1| hypothetical protein SCHCODRAFT_78223 [*Schizophyllum commune*	156	1.00E-36
family_634_mult_7	ref|XP_003037950.1| hypothetical protein SCHCODRAFT_71897 [*Schizophyllum commune*	153	6.00E-36
family_463_mult_7	ref|XP_003025977.1| hypothetical protein SCHCODRAFT_114792 [*Schizophyllum commune*	142	4.00E-32
family_484_mult_7	ref|XP_003037942.1| hypothetical protein SCHCODRAFT_63192 [*Schizophyllum commune*	113	9.00E-24
family_471_mult_12	ref|XP_003037896.1| hypothetical protein SCHCODRAFT_63119 [*Schizophyllum commune*	110	6.00E-23
family_366_mult_11	ref|XP_003036564.1| hypothetical protein SCHCODRAFT_63173 [*Schizophyllum commune*	109	3.00E-22
family_615_mult_7	ref|XP_003035169.1| heat-shock protein 90 [*Schizophyllum commune*	84	8.00E-15
family_85_mult_14	ref|XP_003034629.1| hypothetical protein SCHCODRAFT_84866 [*Schizophyllum commune*	84	1.00E-14
family_605_mult_12	ref|XP_003034778.1| hypothetical protein SCHCODRAFT_232049 [*Schizophyllum commune*	82	6.00E-14
family_386_mult_12	ref|XP_003029499.1| carbohydrate-binding module family 12 protein [*Schizophyllum commune*	79	3.00E-13
family_199_mult_106	ref|XP_003027035.1| hypothetical protein SCHCODRAFT_61583 [*Schizophyllum commune*	71	5.00E-11
family_519_mult_12	gb|ABY85444.1| homing endonuclease [*Agaricus bisporus*]	110	5.00E-23
family_560_mult_8	ref|NP_043722.1| hypothetical protein AlmafMp03 [*Allomyces macrogynus*	62	3.00E-08
family_625_mult_8	ref|XP_002394984.1| hypothetical protein MPER_05041 [*Moniliophthora perniciosa*	193	5.00E-48
Ascomycetes			
family_99_mult_62	ref|XP_002484510.1| hypothetical protein TSTA_040370 [*Talaromyces stipitatus*	72	2.00E-24
family_243_mult_54	ref|XP_002484510.1| hypothetical protein TSTA_040370 [*Talaromyces stipitatus*	67	2.00E-17
family_153_mult_53	ref|XP_002484510.1| hypothetical protein TSTA_040370 [*Talaromyces stipitatus*	80	8.00E-15
family_145_mult_49	ref|XP_002484510.1| hypothetical protein TSTA_040370 [*Talaromyces stipitatus*	60	1.00E-14
family_219_mult_44	ref|XP_002484510.1| hypothetical protein TSTA_040370 [*Talaromyces stipitatus*	82	2.00E-14
family_120_mult_60	ref|XP_002484510.1| hypothetical protein TSTA_040370 [*Talaromyces stipitatus*	58	2.00E-11
family_93_mult_55	ref|XP_001383614.1| hypothetical protein PICST_57317 [*Scheffersomyces stipitis*	107	5.00E-30
family_80_mult_49	ref|XP_001383614.1| hypothetical protein PICST_57317 [*Scheffersomyces stipitis*	117	5.00E-25
family_337_mult_29	ref|XP_001383614.1| hypothetical protein PICST_57317 [*Scheffersomyces stipitis*	110	9.00E-25
family_213_mult_55	ref|XP_001383614.1| hypothetical protein PICST_57317 [*Scheffersomyces stipitis*	115	1.00E-24
family_67_mult_76	ref|XP_001383614.1| hypothetical protein PICST_57317 [*Scheffersomyces stipitis*	111	3.00E-23
family_223_mult_40	ref|XP_001383614.1| hypothetical protein PICST_57317 [*Scheffersomyces stipitis*	108	4.00E-23
family_343_mult_10	ref|XP_001386763.1| manganese-superoxide dismutase [*Scheffersomyces stipitis*	74	6.00E-12
family_579_mult_8	ref|XP_002173974.1| phosphomannomutase [*Schizosaccharomyces japonicus*	229	1.00E-58

Gene ID	Alignment	Score (bits)	E value*
family_359_mult_14	ref|NP_587782.1| argonaute [*Schizosaccharomyces pombe*]	100	1.00E-19
family_236_mult_13	gb|AAG43236.1|AF121229_1 aspartic proteinase precursor [*Botryotinia fuckeliana*	262	7.00E-68
family_589_mult_8	dbj|BAF57023.1| aldehyde dehydrogenase [*Aciculosporium take*]	248	2.00E-64
family_629_mult_11	gb|ADG23121.1| elongation factor 1-alpha [*Rhizoplaca chrysoleuca*]	245	1.00E-63
family_574_mult_11	gb|ABU41923.1| RplA [*Dactylellina haptotyla*]	228	3.00E-58
family_548_mult_15	ref|XP_002835328.1| hypothetical protein [*Tuber melanosporum*	226	1.00E-57
family_508_mult_8	ref|XP_001215029.1| 6-phosphogluconate dehydrogenase [*Aspergillus terreus*	185	1.00E-45
family_323_mult_11	ref|XP_003051617.1| predicted protein [*Nectria haematococca*	184	3.00E-45
family_607_mult_7	ref|XP_001912312.1| hypothetical protein [*Podospora anserina*	162	1.00E-38
family_291_mult_7	emb|CBI52309.1| unnamed protein product [*Sordaria macrospora*]	116	1.00E-24
family_602_mult_9	ref|XP_001793500.1| hypothetical protein SNOG_02907 [*Phaeosphaeria nodorum*	107	7.00E-22
family_622_mult_7	ref|XP_001793500.1| hypothetical protein SNOG_02907 [*Phaeosphaeria nodorum*	106	9.00E-22
family_495_mult_6	ref|XP_003022749.1| hypothetical protein TRV_03131 [*Trichophyton verrucosum*	99	1.00E-19
family_415_mult_29	ref|XP_001269594.1| hypothetical protein ACLA_028940 [*Aspergillus clavatus*	87	5.00E-16
family_409_mult_36	ref|XP_710281.1| hypothetical protein CaO19.6835 [*Candida albicans*	87	9.00E-16
family_514_mult_7	emb|CBI57251.1| putative RFA2 protein [*Sordaria macrospora*]	83	1.00E-14
family_503_mult_17	ref|XP_002559320.1| Pc13g08950 [*Penicillium chrysogenum*	79	1.00E-13
family_96_mult_15	ref|XP_961100.1| hypothetical protein NCU03753 [*Neurospora crassa*	59	2.00E-07
family_171_mult_38	gb|EEH16720.1| hypothetical protein PABG_06807 [*Paracoccidioides brasiliensis*	42	2.00E-06
family_135_mult_33	gb|AAB95256.1| RT-like protein [*Venturia inaequalis*]	482	1.0E -134
Other organisms			
family_378_mult_20	ref|YP_203295.1| orf305 [*Rhizopus oryzae*] >gi|57338992|gb|AAW494...	249	2.00E-64
family_460_mult_11	gb|ADG65261.1| malate dehydrogenase [*Rhizopus oryzae*]	65	4.00E-09
family_449_mult_14	ref|YP_203353.1| orf296 [*Mortierella verticillata*] >gi|57545566|...	44	8.00E-09
family_217_mult_76	ref|ZP_06388631.1| hypothetical protein Ssol98_08391 [*Sulfolobus solfataricus*	114	8.00E-24
family_535_mult_10	ref|YP_946576.1| hypothetical protein AAur_0776 [*Arthrobacter aurescens*	75	3.00E-12
family_39_mult_1241	ref|ZP_03294132.1| hypothetical protein CLOHIR_02084 [*Clostridium hiranonis*	64	2.00E-07
family_552_mult_13	dbj|BAI87564.1| UDP-glucose 4-epimerase [*Bacillus subtilis*	96	1.00E-18
family_108_mult_18	ref|XP_762926.1| 4-hydroxybenzoate octaprenyltransferase [*Theileria parva*	97	4.00E-18
family_583_mult_10	ref|YP_002486246.1| hypothetical protein Achl_0154 [*Arthrobacter aurescens*	55	3.00E-06

Gene ID	Alignment	Score (bits)	E value*
family_112_mult_13	gb|ADD85140.1| calmodulin [*Triticum aestivum*]	295	5.00E-78
family_310_mult_15	ref|XP_002888394.1| hexaubiquitin protein [*Arabidopsis lyrata*	234	3.00E-60
family_385_mult_10	ref|XP_001565477.1| 60S ribosomal protein L26 [*Leishmania braziliensis*	167	3.00E-40
family_41_mult_41	gb|ABR25965.1| hypothetical protein [*Oryza sativa* Indica Group]	126	1.00E-27
family_289_mult_21	gb|ABK96247.1| unknown [*Populus trichocarpa x Populus deltoides*]	127	3.00E-27
family_4_mult_14699	gb|ACU14517.1| unknown [*Glycine max*]	117	9.00E-25
family_281_mult_14	dbj|BAB33421.1| putative senescence-associated protein [*Pisum sativum*	54	5.00E-21
family_303_mult_43	ref|XP_001785946.1| predicted protein [*Physcomitrella patens*	102	2.00E-20
family_127_mult_116	dbj|BAA10929.1| cytochrome P450 like_TBP [*Nicotiana tabacum*]	95	3.00E-20
family_105_mult_93	ref|NP_001169136.1| hypothetical protein LOC100382981 [*Zea mays*]	78	4.00E-20
family_38_mult_58	gb|EFN58729.1| hypothetical protein CHLNCDRAFT_48520 [*Chlorella variabilis*	100	8.00E-20
family_351_mult_19	dbj|BAF01964.1| hypothetical protein [*Arabidopsis thaliana*]	67	2.00E-19
family_360_mult_39	ref|XP_003064996.1| predicted protein [*Micromonas pusilla*	98	3.00E-19
family_59_mult_63	gb|ABK96247.1| unknown [*Populus trichocarpa x Populus deltoides*]	100	4.00E-19
family_308_mult_36	emb|CBJ34222.1| conserved unknown protein [*Ectocarpus siliculosus*.	89	1.00E-16
family_151_mult_46	emb|CBJ34222.1| conserved unknown protein [*Ectocarpus siliculosus*	86	1.00E-15
family_356_mult_20	ref|XP_002488936.1| hypothetical protein SORBIDRAFT_1514s002010 *Sorghum bicolor*	86	2.00E-15
family_181_mult_82	emb|CBJ34222.1| conserved unknown protein [*Ectocarpus siliculosu*s	67	6.00E-10
family_105_mult_93	ref|NP_001169136.1| hypothetical protein LOC100382981 [*Zea mays*	78	4.00E-20
family_127_mult_116	dbj|BAA10929.1| cytochrome P450 like_TBP [*Nicotiana tabacum*	95	3.00E-20
family_132_mult_33	ref|XP_003064992.1| senescence-associated protein [*Micromonas pusilla*	67	6.00E-10
family_121_mult_43	ref|XP_002698317.1| PREDICTED: hypothetical protein [*Bos taurus*]	48	6.00E-11
family_301_mult_23	ref|XP_002698316.1| PREDICTED: hypothetical protein [*Bos taurus*]	52	2.00E-14
family_241_mult_46	ref|XP_001895031.1| hypothetical protein Bm1_17870 [*Brugia malay*	61	6.00E-08
family_294_mult_58	ref|XP_001624693.1| predicted protein [*Nematostella vectensis*]	69	2.00E-10
family_352_mult_34	ref|XP_001624581.1| predicted protein [*Nematostella vectensis*]	53	2.00E-12

Gene ID	Alignment	Score (bits)	E value*
family_239_mult_17	ref|XP_002118239.1| predicted protein [*Trichoplax adhaerens*] >gi...	72	3.00E-11
family_172_mult_54	ref|XP_002167681.1| PREDICTED: hypothetical protein [*Hydra magnipapillata*	87	9.00E-16
family_467_mult_10	emb|CAM91787.1| hypothetical protein [*Platynereis dumerilii*]	172	1.00E-41
family_342_mult_34	ref|XP_002723895.1| PREDICTED: hypothetical protein [*Oryctolagus cuniculus*	84	6.00E-15
family_103_mult_51	ref|XP_729762.1| senescence-associated protein [*Plasmodium yoelii*	95	1.00E-19
family_270_mult_33	ref|XP_729762.1| senescence-associated protein [*Plasmodium yoelii*	86	8.00E-19
family_217_mult_76	ref|XP_729762.1| senescence-associated protein [*Plasmodium yoelii*	87	4.00E-18
family_66_mult_79	ref|XP_729762.1| senescence-associated protein [*Plasmodium yoelii*	79	6.00E-16
family_138_mult_44	ref|XP_729762.1| senescence-associated protein [*Plasmodium yoelii*	79	3.00E-13

*E values recorded at $E>10^{-5}$ confidence level.

Table 4. Summary of the distribution of *P. triticina* expressed sequence tag (ESTs) from haustorium-specific and infected tissue.

Expression sequence tag (EST)	Haustorium Specific	Infected Plant
Total number	2,886	650
Sequences associated with other organisms or empty vectors	2,690	406
Sequences associated with *Puccinia* group	196	244
BLASTX hits ($E \geq 10^{-5}$)	92	178
BLASTX fungal origin	68	134
BLAST2GO hits ($E \geq 10^{-6}$)	106	173
BLAST2GO fungal origin	77	147

Table 5. Predicted secreted proteins identified from *P. triticina* haustorium-specific expressed sequence tags (ESTs).

Name	Gene ID	PCR	bp	a.a.	SP [a] Length
Pt3	Contig249	+	180	60	21
Pt12	Contig91	+	204	68	18
Pt27	Contig90	+	189	63	18
Pt58	Contig131	+	831	277	22
Pt59	PTO3325.E06.S6Wu.ptih	-	615	205	23
Pt63	Contig146	+	639	212	19
Pt65	Contig247	-	225	75	20
Pt67	Contig15	-	594	198	24
Pt68	Contig2	+	576	192	24
Pt69	Contig7	+	1029	343	21
Pt70	Contig29	+	627	209	19
Pt71	Contig233	+	570	189	18
Pt72	Contig31	NT	600	200	18
Pt73	Contig97	NT	180	60	18
Pt74	PTO336.C06.S6Wu.ptih	NT	522	184	29

bp: Number of base pairs; a.a: Number of amino acids; SP: Signal Peptide; NT; Not Tested

[a] Predicted signal peptide based on neural networks (NN) and hidden Markov models (HMM) of the SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/; Nielsen *et al*., 1997)

Table 6. Selected candidate effectors. Predicted secreted proteins identified in *P. triticina* haustorium-specific and infected tissue expressed sequence tags (ESTs).

No	Name	Contig ID	PTT ID[a]	bp	a.a	NLS[b]	Rep. Seq.[c]	Cys Res.[d]	SP Length[e]	SP Score[e]	
										Mean S	HMM
1	Pt3	249	PTT Open Reading Frame	180	60	-	-	5	21	0.932	1
2	Pt12	91	PTTG_09175.1 hypothetical protein	204	68	-	-	1	18	0.910	1
3	Pt27	90	PTTG_00311.1 hypothetical Protein	189	63	-	-	1	18	0.915	1
4	Pt51	-	PTTG_06577.1 hypothetical protein	624	208	-	-	9	20	0.898	1
5	Pt58	131	PTTG_00152.1 predicted protein	831	277	-	-	3	22	0.797	0.994
6	Pt63	146	PTTG_05773.1 hypothetical protein	639	212	-	-	2	19	0.871	0.998
7	Pt68	2	PTTG_04104.1 hypothetical protein	576	192	-	-	5	24	0.946	0.954
8	Pt69	7	PTTG_01757.1 hypothetical protein	1029	343	-	-	1	21	0.746	0.992
9	Pt70	29	PTTG_05971.1 predicted protein	627	209	-	-	-	19	0.852	0.996
10	Pt71	233	PGTG_08705.2 predicted protein	570	189	-	-	12	18	0.639	0.894

bp: Number of base pairs; a.a: Number of amino acids; Cys Res.; Cysteine residues; SP: Signal Peptide

[a] PTT ID: ID in the Puccinia genome database (http://www.broadinstitute.org/annotation/genome/puccinia_group/MultiHome.html)

[b] NLS (Nuclear localization Signal; http://cubic.bioc.columbia.edu/cgi/var/nair/resonline.pl)

[c] Screen of repetitive elements (*http://www.girinst.org/censor/index.php*)

[d] Number of cysteine residues in the putative secreted protein

[e] Length of the predicted signal peptide based on neural networks (NN) and hidden Markov models (HMM) of the SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/; Nielsen *et al*., 1997)

Table 7. Putative functional annotation assigned by BLAST2GO for *P. triticina* candidate effectors

No.	Name	Contig ID	BLAST2GO [a]	Mean E Value [b]
1	Pt3	249	NA	-
2	Pt12	91	NA	-
3	Pt27	90	NA	-
4	Pt51	-	NA	-
5	Pt58	131	Cell wall glucanase	4.46E-51
6	Pt63	146	Hypothetical protein SCHCODRAFT_82504 [*Schizophyllum commune* H4-8]	1.29E-06
7	Pt68	2	Potential secreted cu zn superoxide dismutase	2.37E-50
8	Pt69	7	Predicted protein [*Botryotinia fuckeliana*]	4.43E-07
9	Pt70	29	Hesp-379-like protein	2.11E-37
10	Pt71	233	NA	-

Figure 2. Distribution of *P. triticina* haustorium-specific expressed sequence tag by gene ontology (GO) term assignated by BLAST2GO analysis. X=Predicted function Y= Number of sequences.

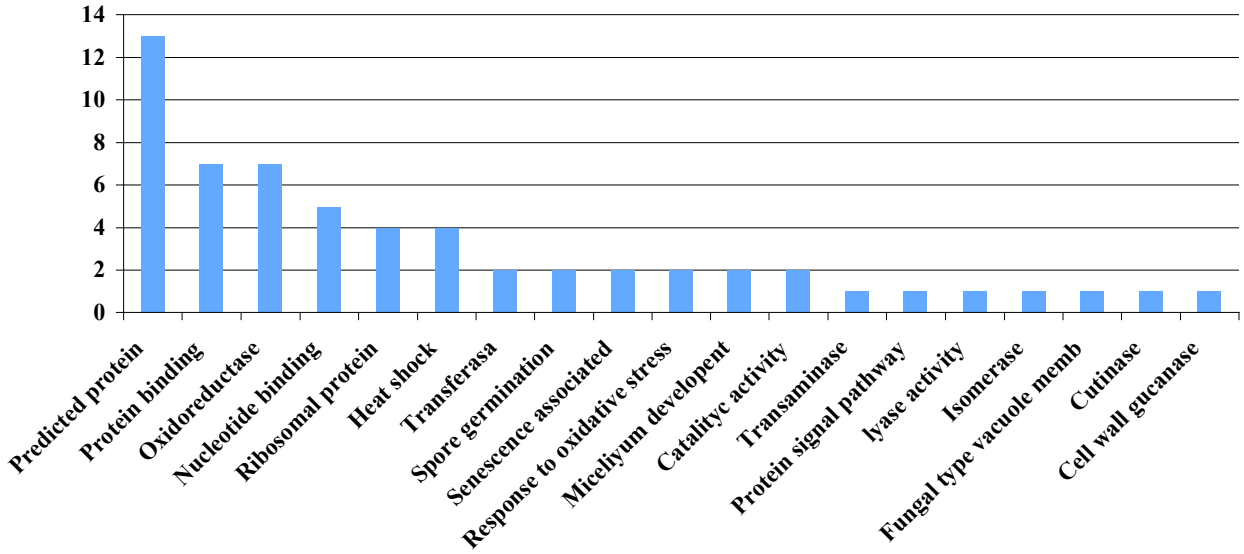


Figure 3. Distribution of *P. triticina* infected tissue (isolate BBBD) expressed sequence tags by gene ontology (GO) term assignated by BLAST2GO analysis. X= Predicted function. Y= Number of sequences**.**

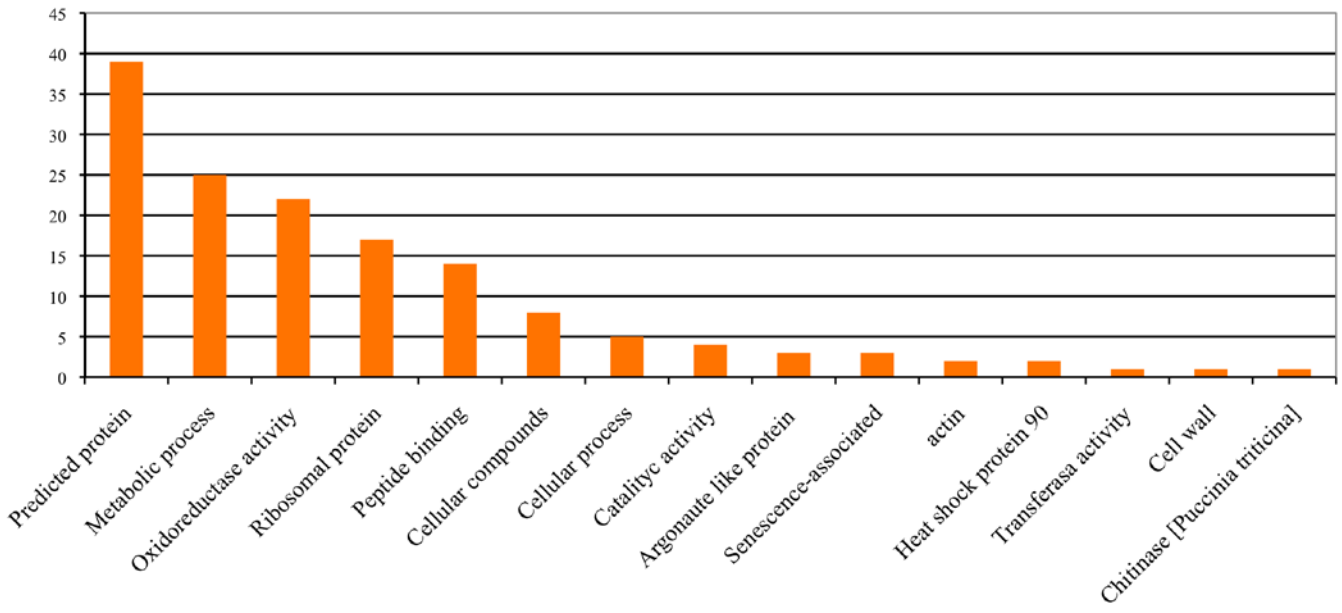


Figure 4. Strategy used for identification of candidate secreted proteins from *P. triticina* expressed sequence tag (EST). ORF: open reading frame. PCR amplification from genomic DNA. RT-PCR in samples harvested 6 days post inoculation (dpi) and germinated spores. SP candidate: secreted protein selected as candidate. *In silico* characterization involves: similarities with Puccinia group database, presence of common domains with effectors from filamentous microorganism, correlation of secreted signal peptide with signal peptide from known effectors and quantification of cysteine residues.

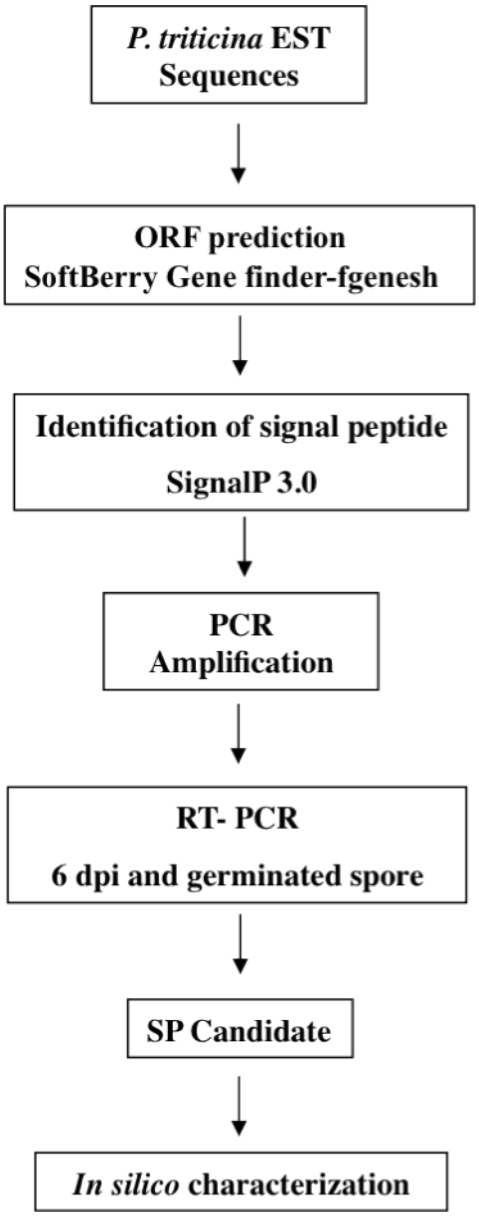


Figure 5. Scatter diagram of scores obtained with SignalP3.0 from annotated Avr genes and Pt effector candidates. HMM score: predicted scores obtained with the SignalP3.0 hidden Markov algorithm. Mean S: obtained with SignalP3.0 neural network algorithm. Scores range from 0 to 1. More confidence in the prediction of the signal peptide with scores near to 1.

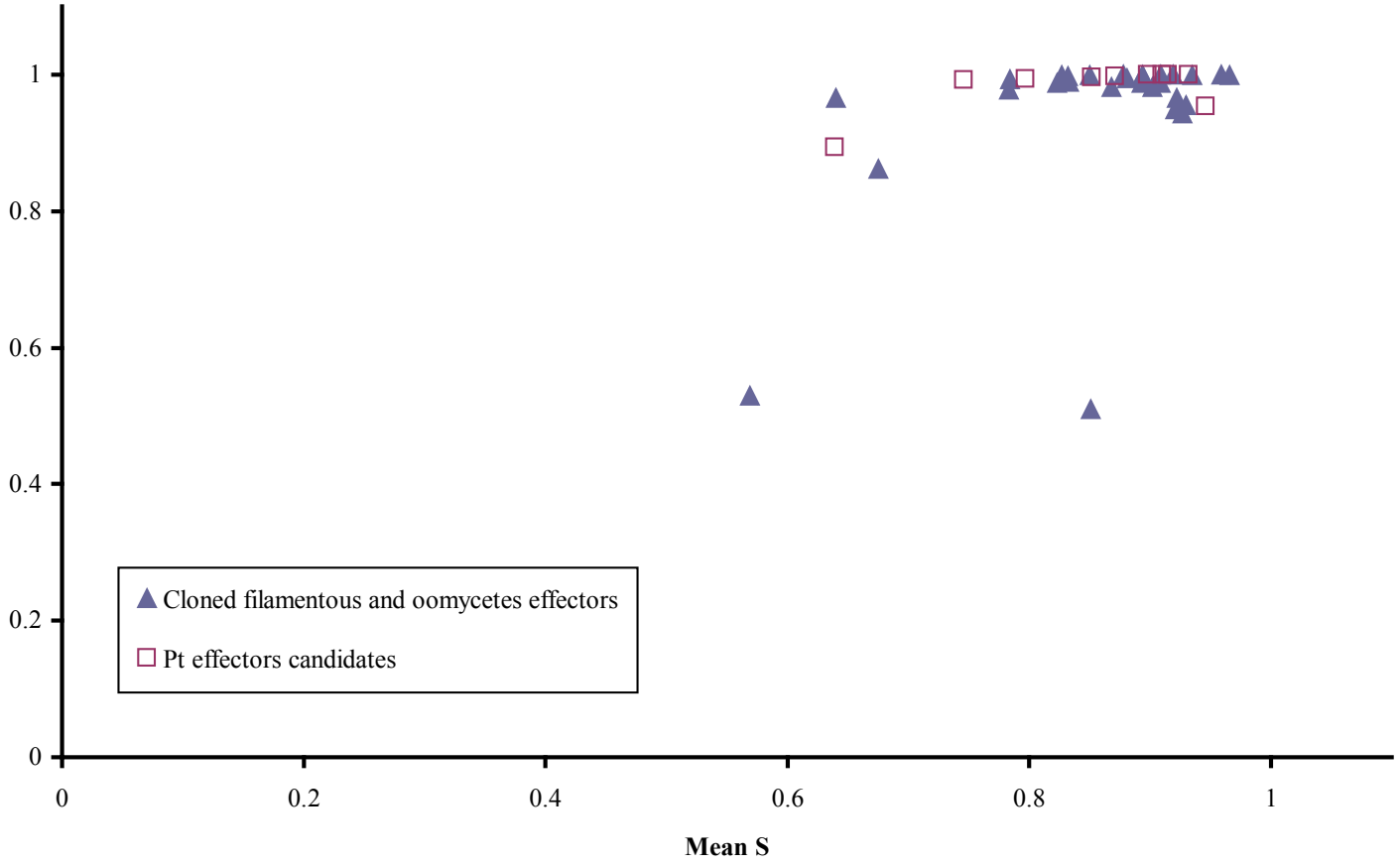
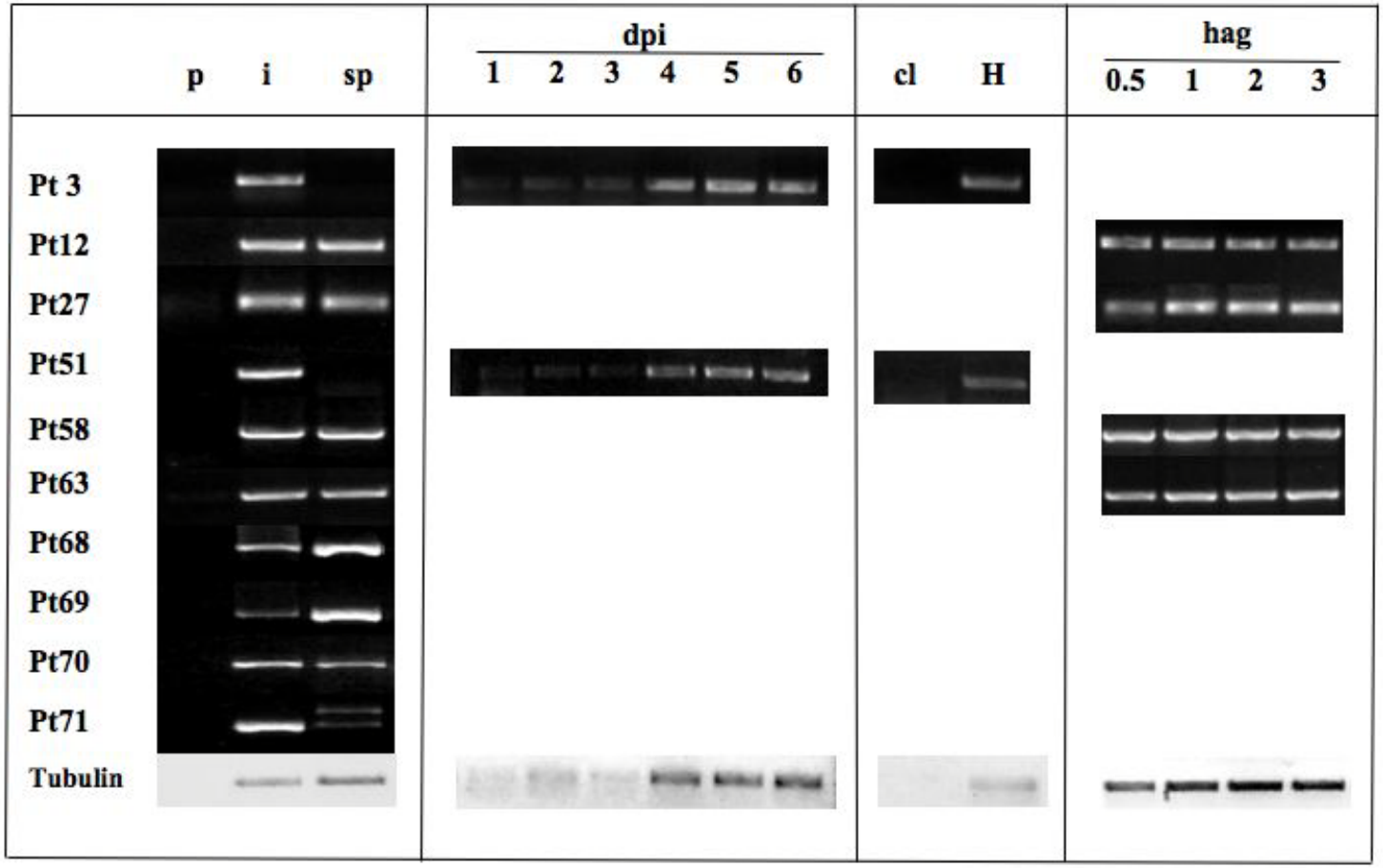


Figure 6. Validation of predicted secreted proteins from *P. triticina* selected as effector candidates using RT-PCR. RNA isolated from non-infected plants (p), plants six days post inoculation (i) and germinated spores (sp). Time course expression was evaluated from 1 to 6 days post inoculation (dpi) and from spores 0.5 to 3 hours after germination (hag). Total RNA from haustorium preparation (H) and mock inoculated plants (cl).



Chapter 3 - **Characterization of predicted secreted proteins from *Puccinia triticina* Eriks. and identification of an avirulence protein.**

V Segovia, H N Trick, K Neugebauer, and J P Fellers

Since Roman times, the *Puccinia* group has caused serious problems in wheat (*Triticum aestivum* L.). Today, resistant varieties are the most cost effective means of control, but the pathogen can evolve and overcome resistance in a relatively short period of time. As an illustration, a new stem rust (*Puccinia graminis f. sp. tritici*) isolate, Ug99, was identified in Uganda in the year 1999 that overcame the resistance gene *Sr31*, which was common in Ugandan wheat varieties (Pretorious *et al*., 2000). In the U.S., Kansas is the leader in wheat production, and leaf rust (*Puccinia triticina* Eriks) is the most severe disease affecting grain production. The fungus is an obligate biotrophic pathogen that needs an alternate host for sexual reproduction. However, in the absence of that host, asexual urediniospores are cyclically produced, dispersed by the wind, and can cause serious epidemics. Urediniospores of leaf rust will land on the leaf surface and germinate. The germ tube will find a stomate by thigmotropism, form an appresorium, penetrate and begin to form a haustorial mother cell. At this point, the fungus will begin to secrete effectors that will alter gene expression in the host cell so that the pathogen can survive. Effectors are responsible for inducing the uptake of nutrients and also inhibit host defense responses (Catanzariti *et al*., 2010). Plants have evolved a single gene defense network that can recognize specific effectors in a gene-for-gene manner (Flor, 1955) and induce plant cell death, thus localizing infection. In wheat, there are more than 67 leaf

rust (Lr) resistance genes that have been named and characterized (Macintosh *et al*, 2010). Most provide resistance in a gene-for-gene manner, though some provide a broad spectrum resistance (Macintosh *et al*, 2010).

Among the biotrophs, the best-characterized pathosystem is flax (*Linum usitatissimum* L.)-flax rust (*Melampsora lini* E.). Flor (1955) was the first to show that the resistance in flax and the incapability to infect in rust strains were genetically based in the plant and in the pathogen. Wheat leaf rust behaves in a similar manner. Avirulence is either dominant or semidominant and is dependent on the respective host resistance gene (Dyck and Samborski, 1968). This type of resistance is now known as effector triggered immunity (ETI; Jones and Dangl, 2006). With ETI, pathogen effectors are secreted to facilitate infection (virulence role), however, disease resistance proteins in the plant perceive certain effectors and through a cascade, a host response is triggered to prevent disease. Any change in either the effector or the resistance gene will yield a compatible reaction. From filamentous microorganisms there have been numerous effectors cloned and each is unique. Most are small proteins that have a secretion signal at the amino terminal end of the protein (de Wit *et al.*, 2009). Catanzariti *et al* (2006) made cDNA libraries from haustoria of flax rust (*Melampsora lini*) and found secretion signals encoded by 21 out of 429 unigenes, and four of the 21, *AvrL567*, *AvrP123*, *AvrP4* and *AvrM*, mapped to avirulence (Avr) loci.

The identification of four flax rust Avr genes from a haustorium cDNA library demonstrated that genomics could be an effective tool in systems that are recalcitrant to classical genetic methods. Genomic resources are rapidly becoming available for cereal rusts with the release of the *P. graminis* and *P. triticina* genomes. But because they are obligate biotrophs, avirulence gene validation is a challenging task. Transformation by bombardment (Webb *et al*.,

2006) or by *Agrobacterium* (Lawrence *et al*., 2010) can be used, however, these are limited by selection of transformed lines on resistant plants. Alternatively, transient expression experiments in host plants can be used to characterize effectors. Particle bombardment in isogenic lines is frequently used to co-express the candidate Avr proteins along with a reporter gene (Jia, *et al*. 2000; Allen, *et al*., 2004; Ridout *et al*., 2006; Armstrong *et al*., 2005; Rehmany *et al*., 2005, Kaneda *et al*., 2009; Qutob, *et al*.,2002; Dou *et al*., 2008). After bombardment into resistant leaves, the transiently expressed Avr protein will induce cell death via recognition by the R gene present. A reduction of the expression of a reporter gene, such as green fluorescence protein (GFP) or beta-glucoronidase (GUS), is used to quantify the presence of a resistance response (Kale and Tyler, 2010).

The wheat-leaf rust pathosystem is poorly characterized at the molecular level. There are isogenic lines that facilitate the study of pathogenicity of the fungus toward specific *Lr* genes (McIntosh *et al*., 1995) and enable characterization for Avr function in given rust isolates. Characterization of haustoria-secreted proteins may help to understand the mode of action of *P. triticina*. To date, no Avr genes from cereal rusts have been cloned. The identification of a leaf rust Avr gene will have a huge impact and may provide information to generate new strategies of disease control. In previous work we identified ten small-secreted proteins. The goal of this research is characterize three of them, Pt3, Pt12 and Pt27, and determine if they have an avirulence function.

Materials and methods

Candidate PCR amplification

E. coli bacteria containing pGEMTeasy vectors with Avr candidates were pulled from the haustoria cDNA library and cultured overnight on LB plates containing 100 mg/L ampicillin at 37 °C. Two ml cultures of single colonies were then grown overnight and plasmid DNA preparations were made using the Qiagen miniprep kit (Qiagen, Valencia, CA). PCR conditions were 20 µl reactions containing 2 µl of plasmid DNA (250 ng), 10 pmol of both forward and reverse primers for each candidate, 2.0 µl 10X Taq reaction buffer (Sigma), 2.5 mM $MgCl_2$, 2.5 mM dNTPs, and 1 µl of Taq enzyme (Sigma). Amplification conditions on the MJ Research PTC100 consisted of 3 min 92 for 3 min, then 35 cycles of 92 °C for 1 min, 60 °C for 2 min, and 72 °C for 2 min, and one cycle of 72 for 10 min. The amplicons were ligated into a TA vector pCR2.1 using the TA cloning kit (Invitrogen, Carlsbad, CA) following manufacturer instructions.

Cloning into pAHC17

The plant expression vector pAHC17 (Chirstensen and Quail, 1996) was used for transient expression of the candidate avirulence factors. The genes were initially PCR amplified as described above, ligated into the pCR2.1 TA cloning vector (Invitrogen, California) and transformed into INValphaF *E. coli* cells. Inserts were cleaved from the plasmid using 1µg of plasmid DNA, 2.5 ul of 10X Restriction Buffer A (American Allied Biochemical, Aroura, CO), and 10,000 units of *Bam*HI. Inserts were gel purified using the Gel Extraction Kit (Qiagen).

pACH17 was prepared using the Qiagen miniprep kit and digested with *Bam*HI as described above. The plasmid was gel purified from a 1% agarose gel by excising the band and using Qiagen gel extraction kit, following the manufacturers instructions. Digested plasmid was treated with 1 unit of calf intestinal alkaline phosphatase (CIAP; Invitrogen, California) and incubated at 37 °C for 5 min, then inactivated by incubation at 65°C for 15 min. To remove CIAP, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and centrifugation at 14,000 *xg* at room temperature for 5 min and the upper phase was transferred to fresh tube. The DNA was precipitated with 0.1 volumes of 3 M sodium acetate pH 5.2 and 2.5 volumes of 100% ethanol. The mix was vortexed and centrifuged at 14,000 *xg* for 5 min at room temperature. Candidate amplicons were cloned into the *Bam*H1 site using T4 DNA ligase (Invitrogen, California), following the manufacturer's instructions. Ligations were introduced into One shot Top Ten® competent cells (Invitrogen, California) using the chemical transformation protocol described by Invitrogen (Cat. # c4040-10). The expression vector with candidates were sequenced to verify correct sequence and orientation.

Transient expression in isogenic Lr lines

In order to verify Avr function in candidate genes, putative candidates were co-bombarded with the reporter gene ß-glucoronidase (*gus*) into Thatcher isogenic lines carrying resistant genes *Lr 9, Lr 24, Lr 26,* and *Lr52,* respectively, and the hard red winter wheat variety Overley carrying *Lr41*. Thatcher (TC) was used as a control. *In vitro* grown plants were used as a source of tissue. Seeds were sterilized with 96 % ethanol for 1 min and washed 1 time with sterile ddH_20 for 1 min. The seed was then treated with a 20 % hypochlorite solution for 20 min followed by three washes with sterile ddH_20 of 1 min each. Seeds were dried overnight at room temperature in a laminar flow hood and stored at 4ºC. Pre-germination of the seeds was

necessary to coordinate the time of germination. Twenty seed were placed into a Petri-dish containing a sterile ddH_20 soaked Whatman 1 paper (90mm). Seeds were kept at 4 ºC for 48 hours and subsequently transfered to room temperature for 2 days. Seeds were placed on the surface of ½X Murashige and Skoog (1962) agar solution (Murashige and Skoog salt 2.15 g /L (SIGMA-Aldrich); sucrose 15g/L; phytagel 1 g /L; pH 5.7) contained in a 25x150 mm glass test tube (Fisherbrand). Tubes were placed in a growth chamber at 21 ºC and 16-hour period and photon flux density of 145 mol m-2 s-2. After two weeks, the first expanded leaves were cut into 10 cm long explants and cultured for 48 hours into a petri dish containing 1/2MS medium, with conditions previously described.

Particle bombardment

DNAdel gold carrier particles, from Seashell Technology (La Jolla, California), were prepared by taking 30 µl of DNAdel™ Gold Carrier Particles (S550d) and mixing them with 20 µl of binding buffer provided by the kit, for a final concentration of 30 mg/ml of gold particles. 2.5 µl of pAHC27-GUS (1µg/µl) and 2.5 µl of candidate plasmid (1µg/µl) was added to the gold suspension for a total volume of 55 µl. 55.5 µl of precipitation buffer was added and incubated a room temperature for 3 min, followed by a centrifugation at 13,000 *xg* for 10 sec in a microcentrifuge. The supernatant was discarded and the pellet was washed with 500 µl of 100% ethanol and vortexed. The suspension was centrifuged again, the ethanol discarded, and the pellet was resuspended by adding 75 µl of 100% ethanol. The solution was sonicated for 10 minutes to break up the gold clumps (Barnstead, Lab-Line, Aqua Wave 9377, St. Louis, MO) and 7.5 µl of the suspension was placed onto a macrocarrier disk and allowed to air dry.

Two pre-cultured leaves 10 cm long were placed in a petri dish containing a wet filter paper and they were held in place by an aluminum disc (Appendix C). The Bio-Rad PDS-1000/He particle gun device (Bio-Rad Inc., Hercules, CA), modified with a barrel attachment to better target the tissue (Torisky *et al*., 1996), was used for bombardment. The petri dish containing the two leaves was placed on the stage at 9 cm of distance. The chamber vacuum was at 25 in Hg and rupture disks of 1100 p.s.i were selected. After bombardment, the leaves were again cultured in petri dishes containing 1/2MS medium for 48 hours in a growth chamber as described above. The experimental design was two leaves per shot, 10 shots for the resistant line and 10 shots for the Thatcher control, per avirulence candidate, and the experiment was repeated twice.

Histochemical GUS staining

Bombarded leaves were cut in 2 cm long pieces that included the bombarded area and placed in GUS buffer (100 mM Na phosphate buffer pH 7.0 with 0.5% Trition X-100, 10 mM EDTA, 0.5 mM of X-gluc, 0.5 mM potassium ferricyanide and 0.5 mM potassium ferrocyanide) and incubated at 37 ºC overnight. Chlorophyll clearing was accomplished by submerging the leaves in 96% ethanol for 24 hours and incubating at 37 ºC. Once cleared, the leaves were scanned and GUS expression quantified by counting blue spots and by measuring the percentage of area of expression with the image analyzer software ASSES 2.0 (APS press). Analysis of variance for both quantifying methods was performed using PROC mixed (SAS 9.1, 2003) and differences were evaluated at α=0.05 of significant level with a model as follow $Y= ß+\alpha+ E_i$ where Y is the media for a given Pt candidate; ß is the number of times the experiment was repeated; α is the effect of Lr gene; E_i is the experimental error (Appendix D).

Results

No protocol has been reported for transient expression of avirulence factors in wheat leaves. Therefore, a protocol for using the particle gun was developed (data not shown). The GFP reporter gene was initially tested and was not effective because of issues with autofluorescence in wheat leaves. Therefore, beta-glucoronidase was chosen. *In vitro* plants were used because a thin cuticle was needed for optimal GUS expression. Since *P. triticina* race PBJL induces a strong hypersensitive response in Thatcher isogenic lines *Lr9, Lr24, Lr26* and *Lr52*, and in the variety Overley carrying *Lr41* (Figure 7), detached leaves from *in vitro* plants were subject to bombardment with only *UidA* (GUS). All of the lines had similar numbers of GUS expressing cells (data not shown).

Three of ten candidates, Pt3, Pt 12 and Pt27, were selected and cloned into plant expression vector pACH17 as *Bam*H1 digested products and expressed under constitutive plant Ubiquitin (Ubi) promoter. Expression vectors were sequenced to verify correct orientation and sequence. Vectors containing Ubi::*UidA* and Ubi::*Pt candidate* were co-bombarded into detached leaves from selected *Lr* isolines (Figure 7). Thatcher was used as a control in each experiment as it is the genetic background of the isolines. Two leaves from each *Lr* line and two leaves from TC were subject to particle bombardment and replicated 10 times each. In total, forty explants, 20 from the *Lr* isoline and 20 from Thatcher were used.

Two methods of quantification were applied. One method was simply to count the number of blue spots. The other method used imaging technology to measure the area of blue color in the leaf. Both sets of data were analyzed. To normalize the data, the data was transformed to log+1. Over all, variance analysis for both blue spots and area showed significant

differences between the Pt Avr candidates ($p < 0.05$). In the interaction of Pt27-*Lr26* and the Pt27-*Lr52*, there was a significant reduction ($p = 0.0026$ and $p = 0.003$, respectively) in the number of blue spots. Measuring percent area also showed significant reductions (0.005 and 0.009, respectively) (Figure 8 and 9). No significant differences ($p > 0.05$) where found in interactions of, *Lr9*-Pt12, *Lr9*-Pt27, *Lr 24*-Pt27, *Lr26*-Pt3, *Lr41*-Pt27and *Lr52*-Pt12. There is discrepancy in the two analysis for both quantification methods in the interactions *Lr9*-Pt3, Lr24-Pt3, *Lr24*-Pt12, *Lr 26*-Pt3 and *Lr52*-Pt3 (Table 8). *Lr26*-Pt27 and *Lr52*-Pt27 were selected to repeat the experiment two more times and corroborate the previous observation. *Lr52* seeds had a high level of contamination, which caused a decrease in the viability of the seed and the experiment could not be done. Observations in 80 explants (40 *Lr26* and 40 TC) were quantified as described before. A decrease in the number of blue cells was observed compared with the expression in Thatcher after co-bombardment of Pt27 and *UidA* gene (% area $p = 0.0005$ and spots $p < 0.0001$; Figure 10 and 11).

Discussion

Some of the most devastating diseases in cultivated crops are caused by biotrophs like downy mildews, powdery mildews and rusts. But, limitations in genetic variability, the lack of new sources of resistance, and the incompatibility of wild species and domesticated varieties makes it difficult to find new resistance. The validation of effector function is a key task to understanding the biology of the molecular interaction in the pathosystem, but intrinsic limitations in the biotrophic nature complicates analysis. Transient expression experiments have been used to characterize effectors *in planta* (Kale and Tyler, 2010; Kaneda *et al*., 2009; Dou *et al*., 2008; Qutob, *et al*., 2002). Particle bombardment and co-expression of effectors with a reporter gene has been successfully used to validate Avr function of pathogen secreted proteins, based on the recognition of Avr candidates by R proteins, and association of reductions in the expression of a reporter gene as indicator of localized cell death. This approach was used to identify ATR1 (Rehmany *et al*., 2005) and ATR13 (Allen *et al*., 2004) from *Hyaloperonospora arabidopsidis*, AVR3a from *P. infestans* (Armstrong *et al*., 2005), AVRk1 and AVR10 from *Blumeria graminis* f. sp. *hordei* (Ridout *et al*., 2006) and AvrPita from *M. oryzae* (Jia, *et al*. 2000). Although it is an important strategy for pathogens lacking of a mutational approach, the variability among the experiments is very high and efficiency of the transformation can vary between bombardment events (Rehmany *et al*., 2005; Jia *et al*., 2000). In this study, two different methods were used to quantify blue cells expressing GUS in an attempt to minimize the variability and achieve confidence in the analysis. Our research shows that this approach can also be used in wheat-rust Avr interactions.

Both percent area and counting blue spots showed a reduction in GUS for two interactions *Lr52*-Pt27 and *Lr26*-PT27. This indicated that Pt27 is putative avirulence factor. Unfortunately *Lr52* –Pt27 experiment cannot be repeated because of limitation in plant material and restriction of time to produce new seeds. The interaction *Lr26*-Pt27 showed a dramatic decrease in the number of cells expressing GUS in both analyses, either counting blue spots or quantifying blue area (Figure 10-11). Further analysis to corroborate the Avr nature of Pt27 in *Lr26* needs to be done. Sequence differences in an *Lr26* virulent race containing a copy of Pt27, or even the presence of a non functional copy needs to be investigated. Leaf rust resistant gene *Lr26* is a seedling resistance gene that shows HR at early stages of infection and controls disease in *Lr26* avirulent races. Interestingly, virulence to *Lr26* appeared shortly after the release of cultivars containing the gene (Pretorious *et al*., 1990; Long *et al*., 1989; Statler, 1985). Although the dynamics of the leaf rust virulent populations and how new pathotypes evolve is not clear (Kosman *et al*., 2004), *Lr26* virulence races could be explained by a mutation in the Avr gene recognized by *Lr26* (Kosman *et al*., 2004).

Candidate Pt27 is a small-secreted protein, with sequence similarity in *P. triticina* (PTTG_00311.1) and does not have a homolog in *P. graminis*. Additionally, there is no alignments with known proteins in GenBank (Table 7). Such unique features support the potential Avr nature. It is possible that *P. triticina* has evolved Pt27 to guarantee successful invasion into the host. Also, it could be involved specifically with leaf colonization rather that infection process in the stem as *P. graminis* does. The signal peptide scores are close to one (0.915/1) which gives some confidence about the translocation of Pt27. Pt27 only has one cysteine residue similar to AVRL567 (Catanzariti *et al*., 2006) from flax rust, which also have one cysteine residue. In flax, several R genes can recognize the same Avr protein from flax rust,

as is the case for AVRL567, recognized by flax resistant genes *L5*, *L6* and *L7* (Dodds *et al*., 2004). Similarly, Pt27 show indications for being recognized by *Lr26* and *Lr52*. But further analysis in *Lr52* needs to be done to have a better understanding of *Lr52*-Pt27 interaction. Candidate Pt12, is also a small secreted protein with one cysteine residue, but transient expression experiments showed no difference. Although Pt3 is a cysteine-rich small secreted protein with high SP scores, no similarity with known proteins in the data base, and is expressed specifically in the haustoria (Figure 6), it does not show a decrease in the number of cells expressing GUS in the isolines evaluated. That might suggest a virulence factor that needs to be further characterized.

Conclusion

From three *P. triticina* secreted proteins tested for avirulance function, Pt27 induced a decrease in the number of cells expressing GUS, which is associated with the localized cell death after the recognition from *Lr26* protein. Although it is an indirect manner to infer Avr activity, this strategy was used to successfully identify Avr genes in other systems. Still further characterization needs to be done to strongly confirm that Pt27 is Avr26. Sequence analysis for *Lr26* virulent races needs to be done. The candidate Pt3 shows ideal properties for being an effector, but its role in virulence needs to be validated. For candidate Pt12, the observations were ambiguous, thus no conclusive observations were obtained from this experiment.

General conclusion and future work

The data generated in this study provides the first insight into the wheat-leaf rust interaction, and opens a set of strategies that can be used to validate potential candidates. This research confirms that expressed sequence tags (ESTs) are a valuable tool to generate information in complicated pathosystems like rusts. From ten *P. triticina* predicted secreted proteins, three were characterized. Characterization of the seven remaining proteins needs to be done. There is special interest in candidates Pt51, small secreted cysteine-rich protein, expressed specifically in the haustoria; Pt58, a predicted cell glucanase, and Pt70, which encodes a cysteine-rich protein with a predicted domain that is developmentally regulated by a MAPK interacting protein. Further analysis is required to confirm the Avr nature in Pt27 with *Lr26*, and *Lr52*. Other studies include the characterization of genes potentially involved in infections and identified in the EST collection, such as the homologs to a SNARE YKT6, a cutinase and a NMT1 protein from *Uromyces viciae-fabae*, which is specifically induced *in planta*.

Figure 7. Isogenic resistant lines showing hypersensitive response (HR) 11 days after the inoculation with *P. triticina* PBJL isolate.

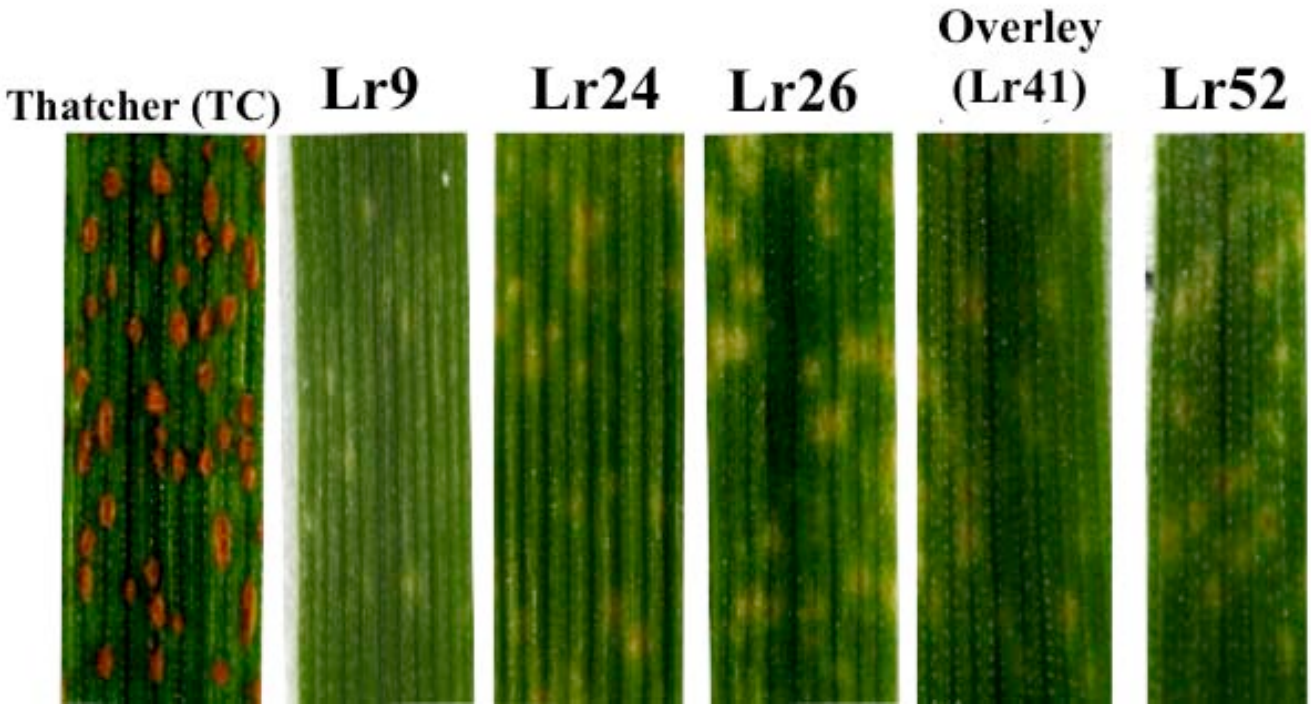


Figure 8. Transient expression experiments using number of blue loci. Pt3, Pt12 and Pt27 candidates were co-bombarded with GUS into Thatcher isogenic lines containing leaf rust resistance genes *Lr9*, *Lr24, Lr26*, and *Lr52* and Thatcher (TC). Gene sequences were expressed constitutively with the Ubi promoter. Overley is a variety with *Lr41* and the control was Overley with empty vector, *Lr41C*. Values correspond to the mean for number of spots in each interaction. ANOVA at α=0.05. Every experiment was repeated two times, with ten replicates. Red Stars: Significant differences. Purple star: Significant differences in both quantification analyses.

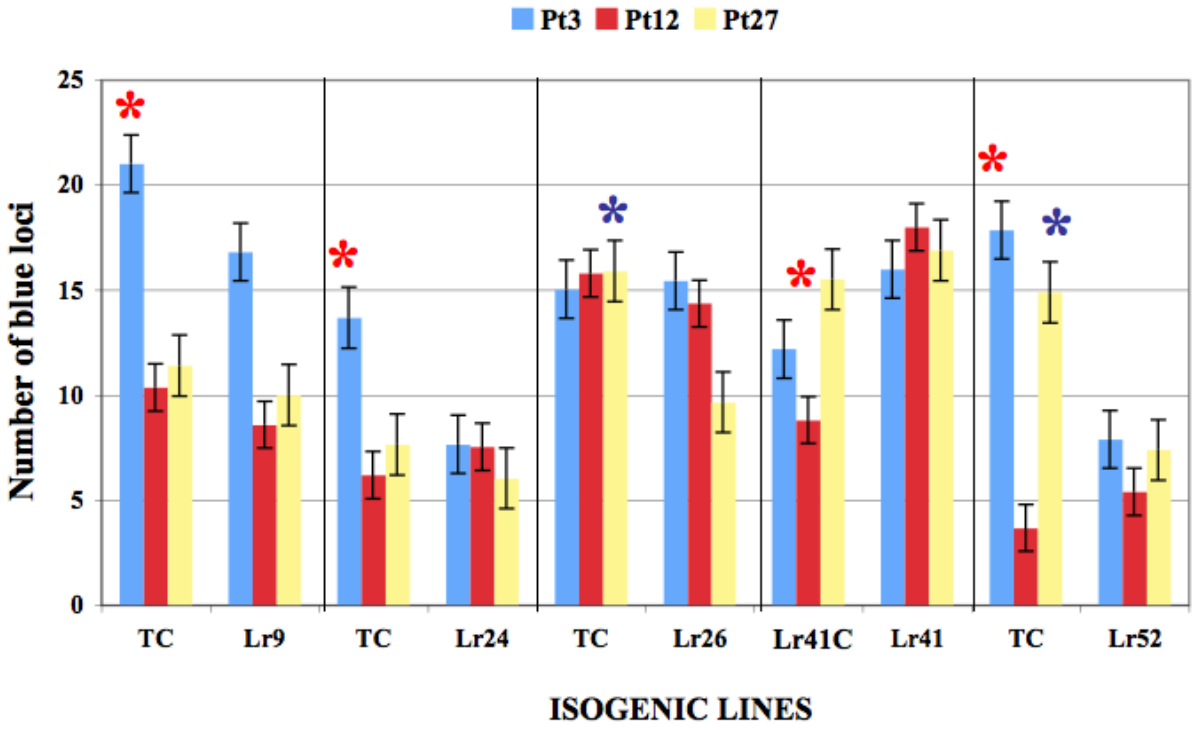


Figure 9. Transient expression experiments quantifying percent area. Pt3, Pt12 and Pt27 candidates were co-bombarded with GUS into Thatcher isogenic lines containing leaf rust resistance genes *Lr9*, *Lr24, Lr26*, and *Lr52* and Thatcher (TC). Gene sequences were expressed constitutively with the Ubi promoter. Overley is a variety with *Lr41* and *Lr41C* the control was Overley with empty vector, *Lr41C*. Values correspond to the mean for number of spots in each interaction. ANOVA at α=0.05. Every experiment was repeated two times, with ten replicates. Red Stars: Significant differences. Purple star: Significant differences in both quantification analyses.

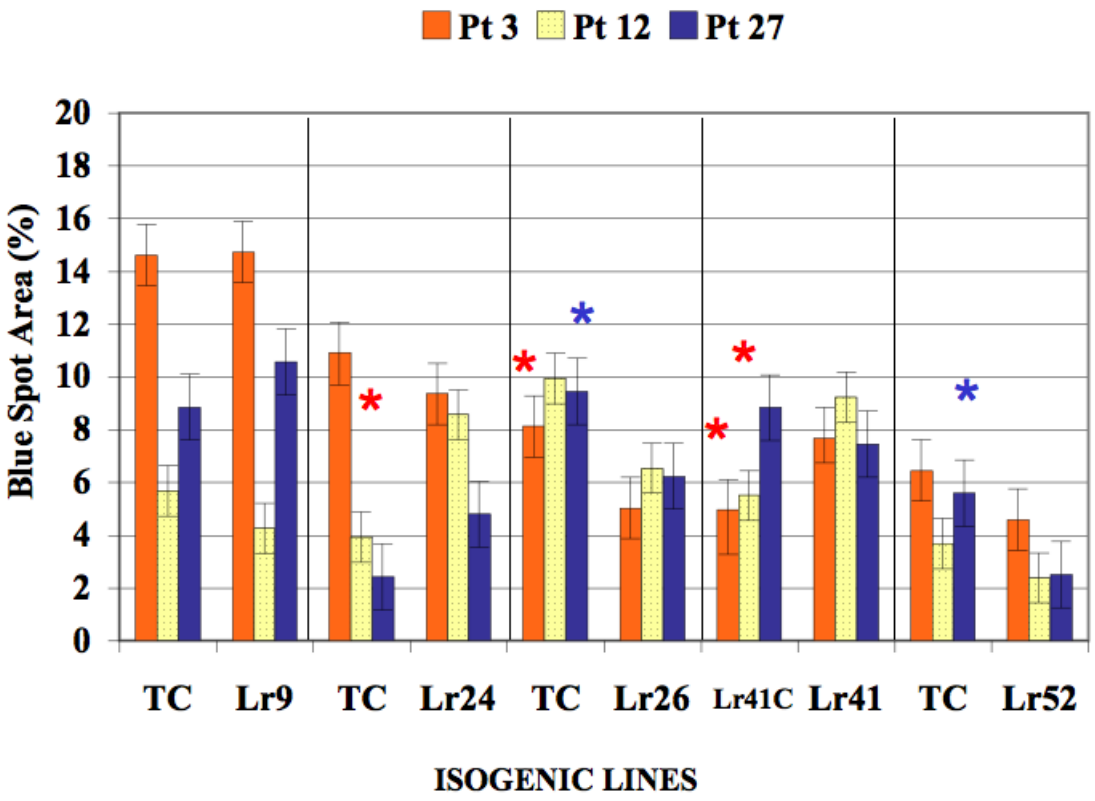


Figure 10. Representative Thatcher *Lr26* and Thatcher leaves showing GUS expression. Leaves were co-bombarded with Ubi::*uidA* in combination either Ubi::Pt27, Ubi::Pt12 or Ubi::Pt27 genes independently. After 48 hours of incubation, leaves were treated for GUS activity and cleared with ethanol.

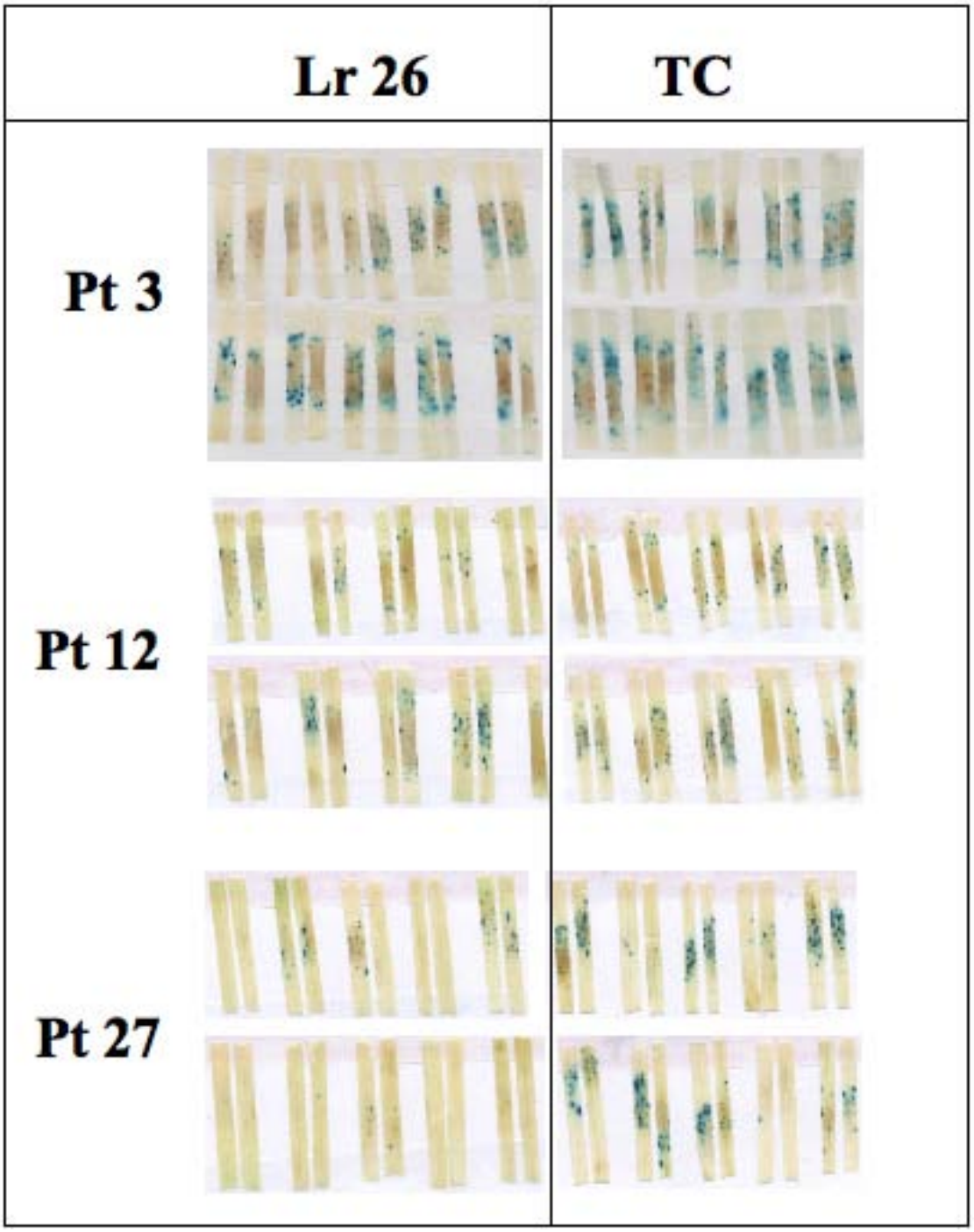
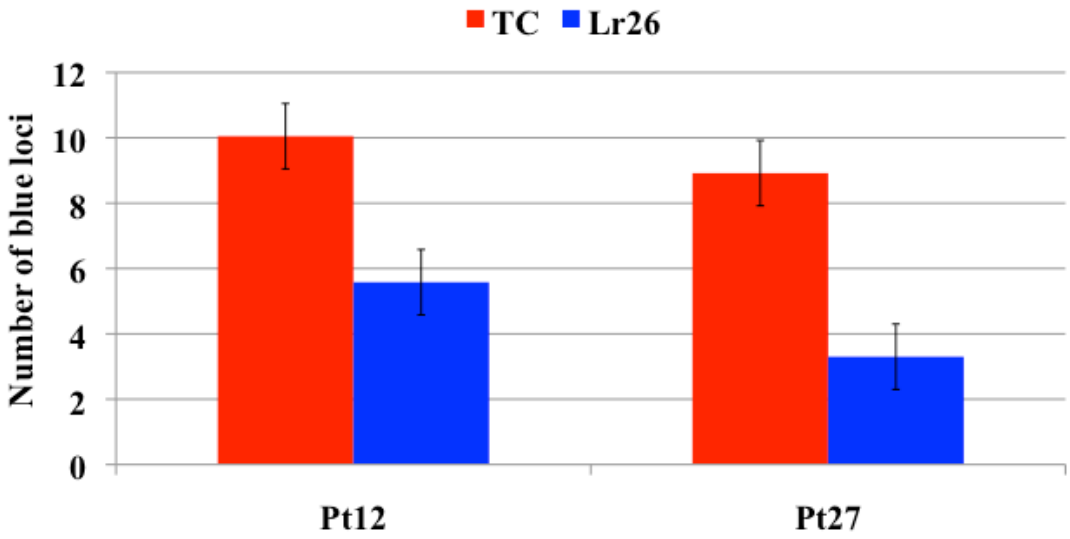
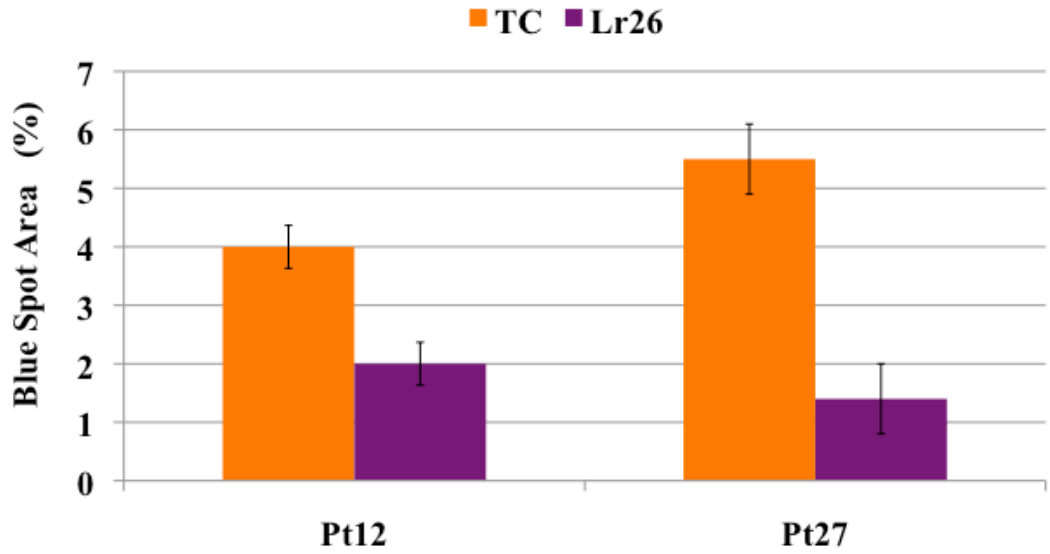


Figure 11. Pt12 and Pt27 candidates were cobombarded with GUS into Thatcher isogenic lines containing leaf rust resistance gene *Lr26* and Thatcher (TC). Gene sequences were expressed constitutively with Ubi promoter. Values correspond to the media for each interaction. ANOVA at α=0.05. Experiment was repeated two times with ten replicates for each line.





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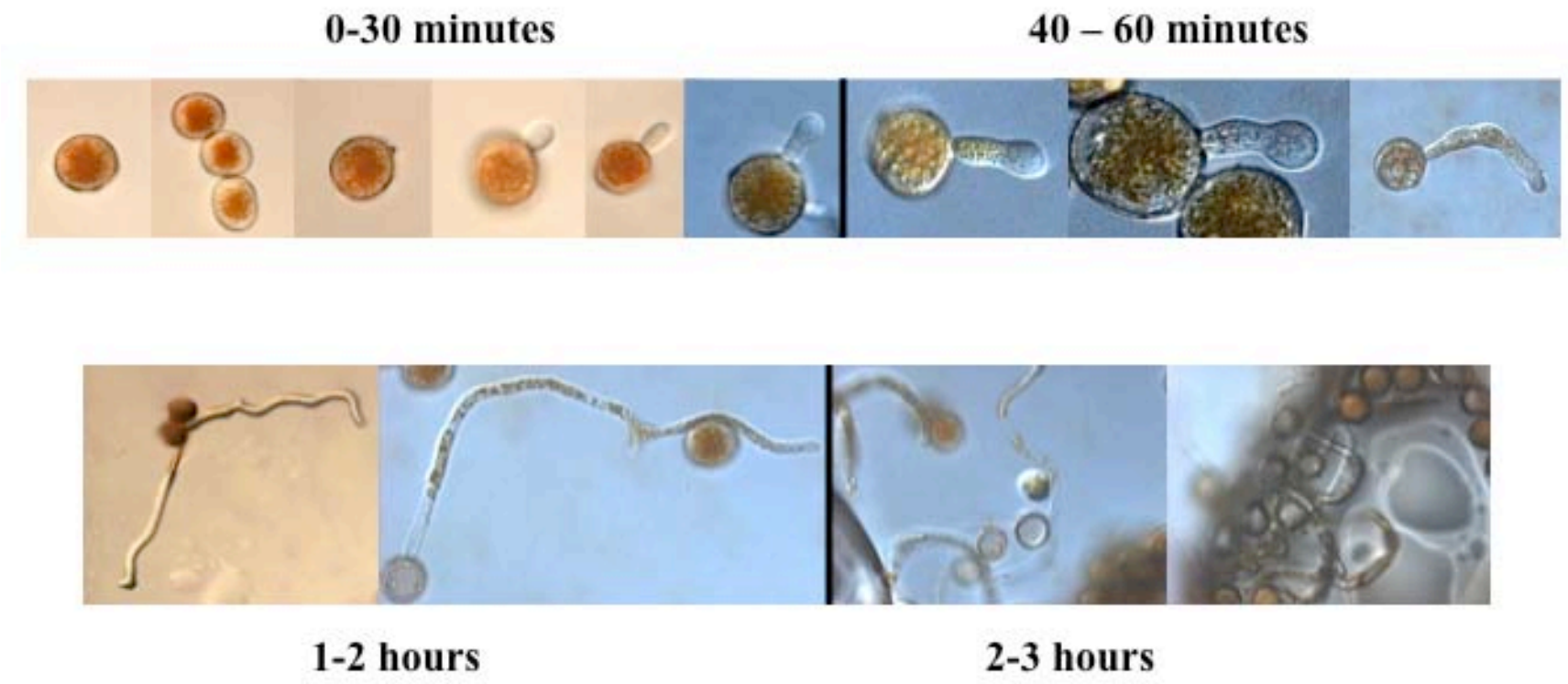
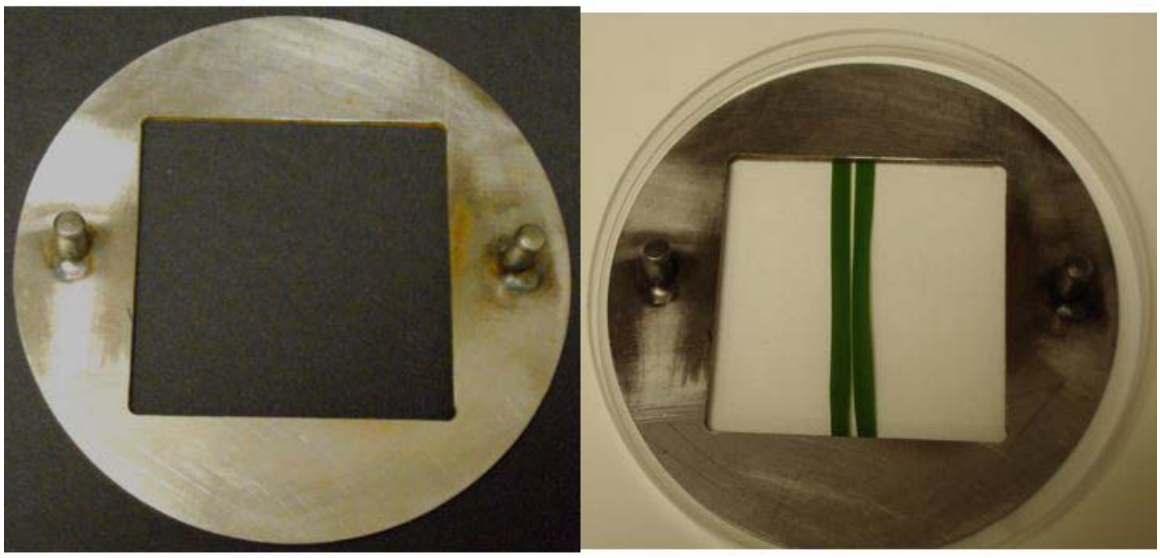
Appendix A - **List of primers used to amplify Pt candidate. Sequence 5'-3'.**

Pt3FW	CAGGATCCATGAAGTGTTCCGTGTTCG
Pt3RW	CAGGATCCCTAGTCTAGGATGTTGTAC
Pt12FW:	CAGGATCCATGCAATTCACCGTTCTCG
Pt12RW:	CAGGATCCTCAGTAGAAGACACCGTAG
Pt27FW:	CAGGATCCATGCAATTCACTACCTTAG
Pt27RW:	CAGGATCCTTACCACCAGCCGTAACGG
Pt51FW:	CAGGATCCTCAGTAGGCTGCGTTCTTT
Pt51RW:	CAGGATCCATGAAAGCTACCGTCGTGG
Pt58 FW:	CAGGATCC ATGAATCGAATCCATTTTT
Pt58 RW:	CAGGATCCTGGTACCTTCGGGCTGCTT
Pt63FW:	CAGGATCCATGACTCCATTCACCAGCA
Pt63RW:	CAGGATCCCCAAACGGAAGCAACGAG
Pt68FW:	CAGGATCCATGCGCTTCTTGAATTTAT
Pt68RW:	CAGGATCCTCAAAGTGTTACAAATCCG
Pt69FW:	CAGGATCCATGTTTCATTTTGGATCTCG
Pt69RW:	CAGGATCCTCAAAGCAAATCGCCTACG
Pt70FW:	CAGGATCCATGCATGCCACCTGCTTTTT
Pt70RW:	CAGGATCCTTAAAGAAGGTTAGTGAGG
Pt71FW:	CAGGATCCATGCAGGTTACTTACTTAG
Pt71RW:	CAGGATCCTTAGGGGCCTTGGAGTGCG

Appendix B - **Identification of critical times for *P. triticina* spore germination**



Appendix C - **Aluminum disc used to hold wheat detached leaves**



Appendix D - **SAS analysis programming**

```
infile 'G:\xlstransientexpression\Book3.csv' delimiter=',';

input ID AVR $    Lr $      REP  SUB      SS $     perc;

tperc=log(perc+1);

run;

proc mixed data=book1;

*where  ID= 1 and Pt='3';

*where ID=2 and Pt='12';

where ID=3 and Pt='27';

class ID AVR Lr rep sub ss ;

model tperc=rep  Lr/outp=respred;

*random rep rep*AVR;

proc print data=diff;where effect='Lr' ;run;

/*proc univariate data=respred plot normal;

var resid;run;*/

 /*media*/

proc mixed data=book1;

*where  ID= 1 and Pt='3';

*where ID=2 and Pt='12';

where ID=3 and Pt='27';

class ID AVR Lr rep sub ss ;

model perc=rep  Lr/outp=respred;

*random rep rep*AVR;

lsmeans Lr/ pdiff adjust= tukey;

ods output lsmeans=mean1;

ods output diffs=diff1;

ods exclude lsmeans;

ods exclude diffs;
```

```
run;

proc print data=mean1; where effect='Lr';run;

ods html close;
```