TOWARDS IDENTIFYING THE PHYSICAL AND MOLECULAR COMPONENTS INVOLVED IN RESISTANCE TO THE WHEAT LEAF RUST PATHOGEN *PUCCINIA TRITICINA* IN WHEAT AND BARLEY

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Towards Identifying The Physical And Molecular Components Involved In Resistance To Leaf Rust Pathogen *Puccinia Triticina* In Wheat And Barley

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ABSTRACT

Genetic resistance is the preferred method to reduce yield losses caused by many diseases including wheat leaf rust. However, in many cases host resistance seems to be short-lived. For decades, scientists have tried and continue trying to understand genetics and physical mechanisms involved in durable host resistance. Incompatible host-pathogen interactions in wheat- *Puccinia triticina* (*Pt*) pathosystem can be classified into pre-and post-haustorial. Post-haustorial resistance tends to be involved in race specific resistance and is commonly characterized by presence of hypersensitive reaction (HR), while pre-haustorial resistance do not generally involve HR. The objective of this work was to understand the physical and molecular components associated with resistance mechanisms to *Pt*. A set of Thatcher near isogenic lines (NIL) carrying different leaf rust (*Lr*) resistance genes and barley (non-host) genotypes were evaluated in time course experiments for histological analysis and relative fungal DNA quantification. Histological evaluation and q-PCR assay showed differences in time of fungal structures formation and amount of fungal DNA among genotypes. The q-PCR assay could differentiate between resistant and susceptible genotypes at 24 hpi. Additionally, *Cq* ratio of q-PCR assay allowed for classification of resistant genotypes carrying pre-haustorial (*Lr*9, Q21860) and those carrying post-haustorial resistance (*Lr*21, *Lr*34 and Harrington). To our surprise, despite carrying pre-haustorial resistance, Tc-*Lr*9 presented HR as early as 6 hpi. Tc-*Lr21* presented HR, before (5 hpi) and after haustoria formation (24 hpi). To better characterize the HR and determine if the HR observed was the product of H_2O_2 accumulation, histochemical and gene expression studies were used. Both methods confirmed that the HR observed in Tc-*Lr9* and Tc-*Lr21* indeed involved H_2O_2 accumulation. In addition, up-regulation of hypersensitive induced resistance genes *TaHIR1*, *TaHIR2* was observed in Tc-*Lr9*. Up-regulation of Tc-*Lr21*

gene at 5 hpi was observed in Tc-*Lr21*. The protocols developed and data obtained from this study provide opportunities for quantitatively assess components of resistance and suggest that some previous assumptions about plant-pathogen interaction in host and non-host systems should be revisited.

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DEDICATION

This Thesis is dedicated to my parents Mr. Bhooma Rao Dugyala and Mrs. Roja Dugyala who believed in me and supported my every decision and encouraged me to be a better person every day.

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CHAPTER ONE: LITERATURE REVIEW

Host: Wheat

Wheat (*Triticum aestivum* L.) belongs to the family Poaceae, which includes other food crops such as rice, corn, barley and sorghum. Wheat is grown worldwide for its grain and is one of the most important staple food crops in developing and developed countries. Wheat provides approximately twenty percent of the calories and protein for more than half of the world's population (Shewry, 2009 and Zare et al. 2015). Wheat is ranked third after rice and corn in total production worldwide. According to U.N Food and Agriculture Organization report, the world population was expected to reach 8.5 billion by 2030, which indicates that global need for food is increasing and production of more wheat will be needed to meet the demand for food.

Evolution

Wheat species belongs to genus *Triticum* and is grouped into three ploidy classes: diploid species (2n = 2x = 14; Einkorn wheat, *T. monococcum*), tetraploid species (2n = 4x = 28; wheat), and hexaploid species (2n = 6x = 42). Of these, the two most economically important species are *T. turguidum* (tetraploid; durum wheat) and *T. aestivum* (hexaploid; bread wheat). The modern cultivated hexaploid wheat comprises A, B, and D genomes (Feuillet et al. 2008). Hexaploid *T. aestivum*, evolved from the cross between the genomes of tetraploid, *T. turgidum* (AABB) and the diploid species known as goat grass *Aegilops tauschii* (DD) (Salamini et al. 2002). *T. turguidum*, the tetraploid (AABB) evolved from the cross of two closely related diploid genomes, *T. monococcum* (AA), and an unknown, possibly extinct, species *Sitopsis* (BB) (Salamini et al. 2002). The origin of hexaploid *T. aestivum* was experimentally confirmed in the 1940s through hybridizations of *T. dicoccum* and *Ae. tauschii*. The resulting hybrids from these crosses were morphologically similar to *T. spelta* and crossed easily with both *T. spelta* and *T.*

aestivum (Faris, 2014). The hexaploid wheat genome contains a total of 17-gigabase-pair (Gb) with approximately 94,000 to 96,000 genes (Brenchley et al. 2012).

Domestication

Domestication of wheat was started about 8,000 to 10,000 years ago in the Fertile Crescent (south-eastern part of Turkey, Syria, Iraq and Iran) (Harlan 1992). The oldest known cultivated wheats were einkorn, a diploid (AA) wheat and emmer, a tetraploid (AABB) wheat (Shewry 2009). Einkorn wheat might have been domesticated from wild einkorn (*T. monococcum* ssp. *aegilopoides*) in the region of Karacadag mountains in Southeastern Turkey (Heun et al. 1997). Domestication of einkorn and emmer wheat for non-shattering rachis led to the efficient harvest of the grain by early farmers without the spikes shattering (Faris, 2014). Later, selection and propagation of diploid and tetraploid wheat for large size kernels, non-shattering and free-threshing traits led to the evolution and domestication of bread wheat (Doebley et al. 2006 and Eckardt et al. 2010). Hybridization between diploid *T. tauschii*, and tetraploid durum resulted in the hexaploid bread wheat that was domesticated and disseminated around the Caucasian region. The Fertile Crescent region of southwest Asia is considered as the center of origin for hexaploid wheat (Brown et al. 2009). Hexaploid wheat appeared abundantly in the Swiss lake area around 2200-1500 BC and in central Europe approximately at 5400-4900 BC (Nesbitt 2001). Current, highly free-threshing, naked bread wheat (*T. aestivum,* AABBDD, 2n = 42) arose from northwest Turkey in the region between the Black Sea and the Caspian Sea before 4000 BC (Dyck et al. 1985 and Huang et al. 2002). Free-threshing bread wheat then spread from to the Nile Basin, central and western Europe, and Asia (Kilian et al. 2010).

Pathogen: *Puccinia triticina*

Wheat leaf rust also is known as brown rust is caused by the biotrophic plant pathogenic fungus *Puccinia triticina* Eriks., (Kolmer et al. 2009). *P. triticina* has a heteroecious, macrocyclic life cycle which means, the pathogen needs two hosts (primary and alternate) to accomplish the entire life cycle (Bolton et al. 2008). Leaf rust has a macrocyclic life cycle which means, all five spore stages can be seen in the life cycle. Leaf rust can be seen worldwide and is considered as the most common and widely distributed, than other rust diseases of wheat such as wheat stem rust (*P. graminis* f. sp. *tritici*) and yellow rust (*P. striiformis* f. sp. *tritici*) (Knott 1989 and Bolton et al. 2008).

Distribution

Leaf rust is the most widely distributed in USA, Canada, Mexico, West Asia, East Asia, Central Europe, Eastern Europe, Middle East, Southern Africa, Northern Africa and Australia (Roelfs et al. 1992, Huerta-Espino et al. 2011 and Kolmer et al. 2009). *P. triticina* spores overwinter in Southern Texas and the US Golf Coast regions, and infect wheat crops are grown as early as February and reach highest severity levels in March and April (Roelfs, 1989). Therefore, Texas is considered as the most important region for the *Puccinia* pathway where urediniospores are carried by the wind to the northern and eastern states including Oklahoma, Kansas and Virginia. Leaf rust infects winter and spring wheat in Minnesota, South Dakota and North Dakota during mid-June to Mid-July (Kolmer et al. 2009). Yield losses in winter wheat can be severe for example, leaf rust epidemics have occurred more frequently on winter wheat in some parts of USA. Yield losses were estimated at more than three million tons with a value of over $350 million from 2000 to 2004 in the US (Huerta-Espino et al. 2011).

Taxonomy and classification

Rust fungi belong to the kingdom of Fungi, the phylum Basidiomycota, class Urediniomycetes, and order urediniales since the rust fungi produce haploid sexual basidiospores on a special club-shaped fruiting body called basidium. Webster, in 1980 placed rust and smut in the class Teliomycetes since, both fungi are similar in basidial structure formation. However, using DNA sequence data, rust and smut fungi were categorized into three classes: Urediniomycetes (rust pathogens), Ustilaginomycetes (smut pathogens), and Hymenomycetes (mushrooms and bracket fungi) (Swann et al. 2001). Considering the morphology of telia and teliospores, originally rust fungi have been categorized into three families: Melampsoraceae, Pucciniaceae, Coleosporaceae (Alexopoulus, 1962). Later, based on sequence variation of ribosomal DNA regions three major sub orders were categorized: Uredinineae, Melampsorineae and Mikronegeriineae (Aime et al. 2006).

The order *Pucciniales* comprises of more than 100 genera and 7000 species (Hawkworth et al. 1995, and McKenzie, 1998). In 1815, Augustin de candolle named wheat leaf rust pathogen as Uredo rubigo – vera (DC). In 1884, Winter placed leaf rust pathogen in *Puccinia recondita*, species complex. Later, leaf rust pathogen was placed in the *Puccinia recondita* f. sp. *tritici* since wheat leaf rust pathogen is considered as a species, distinct from leaf rust on rye and other wheat relatives based on sexual incompatibility of the pathogen (Anikster et al. 1997). The leaf rust pathogen of common wheat and durum wheat at present is designated as *Puccinia triticina* (Bolton et al. 2008).

Epidemiology

Favorable conditions for *P. triticina* infection are similar to the favorable environmental conditions to grow wheat (Singh et al. 1992). Leaf rust urediniospores germination occurs at high

relative humidity with free moisture and temperature ranging from 20°C - 25°C during the day time and 15°C - 20°C at night time are very effective for spore germination (Samborski, 1985) (Table1). Infection can occur within four hours and may produce a new generation of spores every 7 to 14 days after initial infection, if the favorable conditions exist. Dry and windy days followed by cool nights with dew favors the dispersal and germination of spores and may favor leaf rust epidemics (Eversmeyer et al. 2000).

Table 1. Favorable conditions for *P. triticina* infection. (Modified from Singh et al. 1992, Huerta-Espino, and Roelfs et al. 2002).

Leaf rust infection	**Temperature °C**			**Light**	**Free water**
	Minimum	**Optimum**	**Maximum**		
Germination	2	20	30	low	essential
Germling	5	15-20	30	low	essential
Appresorium	-	15-20	-	none	essential
Penetration	10	20	30	No effect	essential
Growth	2	25	35	high	none
Sporulation	10	25	35	High	none

Pathogen infection process

The infection process of *P. triticina* can be divided into three phases, pre-penetration; penetration; and post-penetration (Kolmer et al. 2009). In the pre-penetration phase, under favorable conditions, the urediniospore absorbs moisture, germinates and produces a germ tube within first 4 hours post incubation (hpi). More than one germ tube and branching germ tubes extend from the germ pore but, only one germ tube produces an appresorium in the stomatal aperture of the leaf when recognized by the pathogen. Appresorium with 4-5 lobes successfully forms on the stomata and enters the leaf through the stoma. An infection peg develops from the

tip of the appresorium within 12 hpi and penetrates the stomatal aperture. In the sub-stomatal chamber, the infection peg swells to form a sub-stomatal vesicle (SSV). From the mature appresorium, the contents of the appresorium are transferred into SSV (Kolmer et al. 2009). One or more primary infection hyphae (PH) arise from the SSV upon contact with a mesophyll cell and a septum is formed between the tip of the PH and the SSV, which stimulates the formation of the HMC in a host (Staples and Macko, 1980). After first HMC formation, several secondary hyphae (SH) develops and produces HMC. From the HMC a penetration peg develops which invaginates the mesophyll cell wall and forms a specialized organ, known as haustorium with a slim neck and a body (Heath and Skalamera, 1997) within 18 hpi. Formation of haustorium is the final step in the infection process, and haustorium plays a key role in the transfer of nutrients from host cells to fungal mycelium (Voegele et al. 2011). Haustorium is not completely intracellular because haustorium invaginates and remains separate from the cytoplasm of the host cell by extrahaustorial membrane (EHM) which is mostly derived from the plant cell plasma membrane and extra haustorial matrix (EHMx) (Szabo et al. 2001). EHMx is a gel-like a layer packed mainly with carbohydrates and proteins which lie in between the EHM and the fungal haustorial wall (Voegele et al. 2011). The cytoplasm of haustorium contains two haploid nuclei, mitochondria and other organelles which are separated from the EHMx by a plasma membrane and haustorial wall (Szabo et al. 2001). A collar-like region around the haustorial neck is known as neck-band, and is packed with the electron-dense material (Garnica et al. 2014). Neck-band seals the EHMx so that, no continuation of the cytoplasm of the host occurs into the EHMx (Garnica et al. 2014). Therefore, EHMx remains isolated and separate from the intercellular fungal hyphae and considered as a symplastic compartment, which helps in providing conditions different from the host cytoplasm (Heath and Skalamera, 1997).

Rust haustoria were first isolated from broad bean leaves infected by bean rust fungus *Uromyces fabae* by using a chromatographic method (Mendgen et al. 1992). Later, a haustorial gene expression study was conducted on *Uromyces fabae* (bean rust), by Hahn and Mendgen (1997) which revealed multiple genes involved in nutrient uptake. Later, Voegele et al. (2001) showed that the hexose transporter, HXT1, located in haustorial plasma membrane transports sugars including glucose and fructose and amino acid transporters AAT1, AAT2, and AAT3 transport amino acids into haustorium. In *U. fabae*, a gene was identified and characterized, which is homologous to invertases and aids in cleavage of sucrose into D-glucose and D-fructose (Voegele et al. 2011). The transcriptome analysis of haustoria isolated from wheat stripe rust fungus *Puccinia striiformis*, showed that haustorium actively uptake nutrients from the host plant (Garnica et al. 2013). An electrochemical gradient is caused across the haustorial plasma membrane by H^+ -ATPase, which drives sugars including glucose, fructose and other amino acids into the haustorium from EHMx. Voegele et al. (2011), determined that fructose is converted into mannitol by MAD1p in the haustoria of *U. fabae*. Mannitol might play a role in the storage of carbohydrate in the mycelium, urediniospores and haustoria of *U. fabae* and, mannitol may be used to suppress the host defense responses involving reactive oxygen species in *U. fabae* (Voegele et al. 2005, Voegele et al. 2011). D-sucrose is converted into Arabitol through the glycolysis and pentose phosphate pathway (PPP) and used as carbohydrate storage in the urediniospores. However, it is still unknown how the carbohydrate storages are translocated from the lumen of haustorium to the newly formed spores. Haustoria utilizes the sugars and amino acids and increase in number and further colony develops and forms a uredinium. By 7 dpi completely established uredinium develops and begins sporulation. Mature and newly formed spores can be observed in the uredinium at 14 dpi.

From the recent studies, it is known that haustoria not only involves in exchange of nutrients (Heath and Skalamera, 1997) but also, is involved in production and secretion of effector proteins into the host cytoplasm (Garnica et al. 2014). These effectors not only, involved in the suppression of the host defense responses but also, manipulating the host metabolism to obtain nutrients that support the growth of the pathogen (Voegele et al. 2011). Most of these effectors are small secreted proteins with unknown function that are released into the plant apoplast or cytosol by the pathogen. For oomycetes effectors, the motif RXLR was identified near the N-terminus (Birch et al. 2008). For bacterium effectors, type three secretion system (T3SS) was identified to deliver effectors directly into the plant cytosol (Chang et al. 2005). No common motif has been identified for filamentous fungus effectors. To date, 32 Avr have been cloned most of which are cloned by map-based cloning and reverse genetics (de Wit et al. 2009). The bacterial effector *P. syringae* AvrPto is one of the best-characterized effectors in prokaryotes which involves in flg22-induced FLS2 signaling and inhibits PTI (Xiang et al. 2008). Oomycete effector, AVR1b have been characterized from *Phytophthora sojae* which cause root rot on soybean (Shan et al. 2004). Other effectors *Avr1a, Avr3a* and *Avr3c* have been cloned from *P. sojae* (Qutob et al. 2009). From the tomato pathogen, *Cladosporium fulvum*, an AVR gene has been cloned which produces the effector *Avr9* into the apoplast that encodes 63 amino acid protein (van Kan et al. 1991). AVR9 is recognized by Cf-9, an extracellular LRR-TM resistance protein. From the hemi biotrophic filamentous ascomycete fungus *Magnaporthe oryzae* (rice blast fungus), eight *Avr* genes have been cloned viz., *AvrPita, AvrACE1, Pwl2, AvrPiz, AvrPiia, AvrPii* and *AvrPik/km/kp.* In tobacco AvrPiz-t, R protein has been recognized, in the plant cytosol which suppresses BAX-induced cell death in tobacco (Li et al. 2009). The *Avr* genes *AvrL567,* were cloned from the *Melampsora lini* (flax rust) (Dodds et al. 2004). Even

though some of the fungal effectors, might involve in suppressing the immune responses there might be other effectors that may have roles in manipulating the host metabolism to increase the transfer of nutrients from the host to the pathogen to withstand the infection (Dodds et al. 2004).

Biology and disease cycle

P. triticina is a heteroecious and macrocyclic rust fungus, requiring two taxonomically unrelated host species to complete its life cycle. It requires wheat as a primary host for asexual life cycle and *Thalictrum speciosissimum*, *Isopyrum fumaroides*, *Anchusa* or *Clematis* species as alternate hosts (Knott, 1989) to complete the sexual cycle. Macrocyclic rust fungi produce five different spore stages in their life cycle including basidiospores, pycnidiospores, aeciospores, urediniospores, and teliospores (Bolton et al. 2008).

Urediniospores produced on wheat are carried away by the wind and re-infect wheat when favorable moisture and temperatures of 10–25^{0}C exists on the leaf surface (Roelfs et al. 1992). By the end of the wheat season, urediniospores develop in to, brown, two-celled, smooth walled teliospores which survive the unfavorable conditions (hot, dry summers) (Anikster, 1986). Under favorable conditions, teliospores germinate and produce a basidium. Basidiospores are then formed in the basidium and mature Basidiospores are released under humid conditions and are carried away to alternate hosts by winds (Roelfs et al. 1992). Basidiospores infect the alternate host, germinate and produce pycnidiospores in a flask-shaped pycnia. Pycniospores function as male gametes and receptive hyphae, function as female gametes, (McIntosh et al. 1995 and Bolton et al. 2008). Pycniospores and receptive hyphae fuse to form aecium and aeciospores on the lower side of the leaf, directly below the pycnidium. (McIntosh et al. 1995 and Bolton et al. 2008). Aeciospores can be wind disseminated and infect wheat the primary

host. Under favorable conditions, aeciospores germinate on the primary host, resulting in the production of asexual urediniospores (Knott, 1989).

Signs and symptoms

Leaf rust occurs throughout all growth stages of wheat plants (Bolton et al. 2008). Leaf rust appears on the leaf blades and leaf sheaths. Under favorable conditions, such as high inoculum pressure, and in highly susceptible cultivars leaf rust occurs on stems and heads (Murray et al. 1998). Symptoms begin as small, circular to oval yellow spots (about 1-2 mm in diameter) on infected tissue on the upper surface of leaves. As the disease progresses, the spots develop into orange colored uredinium which may be surrounded by a yellow chlorotic halo. In severe infection cases, the uredinium can spread almost entire leaf surface (Hoffmann and Schmutterer 1999). Uredinium tears the outer epidermal layer of leaf tissue and emerge as pustules where sporulation occurs. Urediniospores can be wind disseminated and infect host plants hundreds of kilometers from their source plants which result in the wheat leaf rust epidemics on a continental scale (Dyck et al. 1985). As the disease progresses, black colored teliospores, may be produced resulting in the black lesions on the leaf. These black spores are known as teliospores, they usually originate from telia which are produced on the leaf. Leaf rust infection causes an increase in transpiration and reduces photosynthetic activity of leaves because of chlorosis. The withering of leaves starts from the leaf tips, and premature defoliation occurs. Therefore, leaf rust infection results in decreased number of kernels per head as well as a lower weight of kernels due to shriveling of kernels (Knott, 1989, Hoffmann and Schmutterer 1999, and Bolton et al. 2008).

Disease relevance for wheat production

Wheat leaf rust is the most common and widely distributed rust diseases of wheat and occurs wherever wheat is grown (Chester, 1946). Wheat leaf rust pathogen primarily attacks leaf blades, leaf sheath and glumes in highly susceptible cultivars. If infection occurs early, 60-70% of yield loses occurs while, if infection occurs after flag leaf emergence it may cause yield loses of up to 30% (Huerta-Espino et al. 2011). Damage due to leaf rust becomes more severe when a single genetically homogeneous cultivars or closely related cultivars are sown in large areas (Samborski, 1985). Losses in grain yield due to leaf rust are mainly due to the reduced floret set because the seed will be mostly shriveled (Roelfs et al. 1992, McIntosh et al. 1995, Kolmer et al. 2007). Under high inoculum pressure, plants might die. Yield loss due to leaf rust infection in US and Canada have ranged from 10-28% in resistant cultivars whereas in hard red winter, soft red winter, hard red spring wheat susceptible cultivars it has ranged from 25-95% in hard red winter, soft red winter, hard red spring wheat (Mains, 1930, Caldwell et al. 1934, Chester, 1939, Peturson et al. 1945 and Johnston, 1931). In developing countries including Mexico, India, Pakistan, Bangladesh and China and in India yield losses of up to 50% have been recorded due to severe epidemics (Hanson et al. 1982 and Joshi, 1976). A severe leaf rust epidemic in 1978 resulted in estimated national loss of $US 86 million in Pakistan and £83 million in Europe (Huerta-Espino et al. 2011). In Australia, leaf rust epidemics caused serious damage in 1880's and estimated crop losses varied from 30% in the leaf rust susceptible cultivars (Rees and Platz, 1975). In the US, loses due to leaf rust were estimated over 3 million tons in 2002-2004 which is worth over $350 million (Huerta-Espino et al. 2011). Kansas, a major wheat growing state in the US, reported yield losses of about 14% in 2007 (Appel et al. 2010).

Management of wheat leaf rust

P. triticina requires a living host to survive from one season to the next season. Leaf rust can be managed by collection and disposal of crop debris thorough stubble working and tillage (Roelfs et al. 1992). Eradication of volunteer plants and removal of the green bridge in some areas minimizes the leaf rust fungus survival and reduces the over wintering of inoculum (Murray et al. 1998, Roelfs et al. 1992). Another method to manage leaf rust is the application of the appropriate amount of fertilizers. For example, excessive usage of nitrogen fertilizers or increased nitrogen content in the wheat leaves increases susceptibility to leaf rust (Robert et al. 2004). Timing, frequency and amount of irrigation also play a role in controlling leaf rust infection in some regions (Roelfs et al. 1992). Adjusting planting dates including delayed winter wheat planting reduces movement of inoculum from nearby fields of spring or late winter wheat cultivars. On the other hand, if the inoculum arrives late in the season, early planting of wheat allows the plants to mature early which results in the escape of leaf rust infections become they become serious (Knott 1989). Overlapping of susceptible varieties of wheat over large areas increases the chances of the build-up of rust spores, leading to early infection in next sown crops with suitable environmental conditions because urediniospores are carried away by the wind through long distances. Leaf rust disease can only be managed by cultural methods to some extent only and cultural practices alone might not effectively control leaf rust because urediniospores are dispersed by the wind across long distances.

Application of foliar fungicides is another strategy to manage rust diseases. There are several fungicides labeled for leaf rust on wheat such as fungicides like mancozeb and metconazole is an effective measure to control rust diseases. However, application of fungicides is expensive and labor intensive. It costs millions of dollars to wheat growers annually and

requires specific application timing such as prior to infection. Protection of flag leaf is critical to reducing yield loses. Additionally, application of fungicide might not be a feasible option, particularly in developing countries due to lack of an effective distribution and infrastructure. Many developing countries depend up on bread wheat as a staple food, but the use of foliar fungicides is not an economic reality. Therefore, one of the important strategy to protect wheat from rust diseases is using resistant cultivars. Genetic resistance to rust diseases has been the preferred method to manage wheat leaf rust over the last 40 years (Singh et al. 2001) since it is environmentally friendly and economical particularly for farmers in developing countries.

Genetic resistance to rusts pathogens

During their life cycle, plants are constantly exposed to both biotic and abiotic stress. In the plant-pathogen interactions, plants are subjected to biotic stresses from insects, nematodes, fungi, oomycetes, bacteria and viruses. Plants tried to defend themselves from the pathogen attack by exhibiting resistance. Resistance has been defined as the ability of the host to retard the growth and development of the pathogen inside the plant tissue (Parlevliet, 1985). Based upon the nature of plant-pathogen interactions, resistance can be categorized into two types: Non-host resistance (NHR) and host resistance.

Non-host resistance (NHR) to rust pathogens

NHR is defined as the resistance of all accessions of one plant species to all isolates of the pathogen and exhibits a uniform level of resistance in all interactions between both species (Heath, 2000). NHR and host resistance are mainly differentiated based on basic compatibility existing between a plant and pathogen (Ellingboe, 1976). In the case of non-adapted pathogens, basic compatibility is the major requirement for successful colonization of plant species by pathogen and to overcome several components of plant defense responses (Bettgenhaeuser et al.

2014). For example, in the event of infection by rust pathogen, into the non-host plant leaves, most of the germinated urediniospores are incapable of finding stomata and form appresorium. However, some of the rust pathogens are capable of forming appresorium on membranes simulating host leaf topography (Heath, 1977 and Allen, et al. 1991).

Non-adapted rust pathogen must also be able to overcome the multiple layers of basal defense response of the plant, which is initiated during the early phases of pathogen attack (Bettgenhaeuser et al. 2014). NHR defense responses include pre-formed physical barriers such as a wax layer, cuticle, and cell walls (Thordal-Christensen, 2003). Pre-formed chemical barriers include the production of antimicrobial secondary metabolites like phenols and alkaloids (Nurnberger and Lipka, 2005). If the pathogen is able to overcome the two layers of pre-formed physical and chemical barriers, then the active defense responses of the plant such as the recognition of a conserved set of pathogen factors, which cannot be modified or eliminated by the pathogen without affecting pathogen fitness, will be triggered. According to Gill, et al. (2015) NHR overlaps with the basal defense response of host since both, non-host and host plant resistance, involves the recognition of similar pathogen factors.

PAMP/DAMP triggered immunity (PTI/DTI), which is triggered by pathogen-associated molecular patterns (PAMP) or damage associated molecular patterns (DAMP) via membrane localized pattern recognition receptors (PRRs), leads to a signaling cascade and suppression of the pathogen growth (Jones and Dangl, 2006 and Ishiga, et al. 2013). Some of the examples of PAMPs include the fungal chitin and xylanases (Zipfel, 2008), bacterial flagellin, elongation factor Tu (EF-Tu), lipopolysaccharides, oomycete elicitor INF-1 (Dangl and Jones, 2001). PTI is not involved in the production of any visible symptoms but is typically involved in the recognition of active defense mechanisms such as PAMPs/DAMPs. This type of non-host

resistance is known as Type I NHR (Zipfel, 2008). For example, in the broad bean- wheat stripe rust pathogen (*Vicia faba-Puccinia striiformis* f. sp. *tritici*) interaction, no visible symptoms were observed on broad bean leaves after inoculation but the fungal growth, including appresorium formation, was observed microscopically (Cheng et al. 2012). Type II NHR leads to rapid hypersensitive response (HR) which results in cell death/visible necrosis (Peart, et al. 2002 and Mysore and Ryu, 2002). HR is triggered in response to effectors released by pathogen and elicits effector-triggered immunity (ETI). For example, a histological and molecular study was conducted by Neu et al. (2003) to understand the barley- wheat leaf rust pathogen (*H. vulgare- P. triticina*) interaction. In this study, barley lines L94 and Bowman infected with *P. triticina*. Microscopic observations demonstrated the presence of secondary hyphae and HMCs in the established colonies, suggesting that *P. triticina* can successfully infect barley lines L94 and Bowman. However, barley lines L94 and Bowman presented cell death at the site of *P. triticina* aborted colonies. Therefore, PTI and ETI might play an important role, not only in adapted pathogen-host interactions, but also in non-adapted pathogen-non-host interactions (Cheng et al. 2012).

A single pathogen species can trigger both type I and type II NHR on different non-host plant species. For example, *P. syringae* pv. *phaseolicola* a pathogen of common bean, triggers type I NHR in Arabidopsis and type II NHR in tobacco. Similarly, a single plant species can exhibit both type I and type II NHR to different non-adapted-pathogens. For example, *N. benthamiana* exhibits type I NHR against *Xanthomonas campestris* pv. *campestris* and type II NHR against *P. syringae* pv *Tomato* (Peart, et al. 2002). Many of the plant defense responses such as production of reactive oxygen species (ROS), lignification, cell wall formation and papillae formation are similar to R-gene (host resistance) responses during an incompatible

interaction and are involved in both host and NHR (Leeshouwers et al. 2000, Huckelhoven et al. 2001, Mysore et al. 2004). NHR is considered as the most common and durable form of resistance since, NHR relies on multiple layers of defense components/mechanisms including basic compatibility, pre-formed physical and chemical barriers, and the exhibition of HR (Nurnberger and Lipka, 2005 and Lipka et al. 2010).

Host resistance to rust pathogens

Host plants have two distinct resistance receptors involved in recognition of pathogen. The first type is pathogen recognition patterns (PRRs), located on the plasma membrane, which recognizes PAMPs or microbe associated molecular patterns (MAMPs) (Qi et al. 2011). PAMPs are small molecules conserved in different pathogen groups for example, chitin for fungus, and flagellin in bacterial flagella, which are recognized by the PRRs and initiate PTI to suppress the pathogen. PTI usually involves ion fluxes (H^+, K^+, Cl^-, and Ca^{2+}), stomata closure, PR protein production and mainly contributes to non-host and basal resistance (Jones and Dangl, 2006). As a result, pathogens have evolved to avoid recognition by the host pathogens might secrete specific effector molecules (Avr proteins) to suppress the defense responses initiated by PTI through the secretion of effector molecules which results in effector triggered susceptibility (ETS) (Hauck, et al. 2003, Jones, et al. 2006). To recognize these effectors, plants evolved a second type of recognition by cytoplasmic R proteins encoded by *R*-genes. The majority of these R proteins belonging to the nucleotide binding-leucine-rich repeat (NB-LRR) family (Luck et al. 2000 and Caplan et al. 2008) which encodes for proteins like the nucleotide binding, leucine-rich repeat (NBS-LRR) proteins, coiled-coil (CC-NBS-LRR) and Toll-interleukin-1-like receptor (TIR-NBS-LRR), recognizes the pathogen-associated proteins (PR proteins) which results in effector triggered immunity (ETI). ETI is specific to the pathogen strain or race usually involves

antimicrobial compounds, cell wall strengthening, and production of pathogenesis related proteins (PR proteins) which lead to hypersensitive reaction or cell death (HR) which can slow down pathogenesis and prevent further spread of a biotrophic pathogen (Dangl and Jones, 2001). Effector recognition in turn, increases the selection pressure on the pathogen, thus eventually leading to no recognition by the plant, and restoring the virulence of the pathogen (Jones and Dangl, 2006). As a result, new plant receptors evolve that can recognize these novel effectors, resulting again in ETI. This coevolution between plants and plant pathogens leads to molecular arm race (Jones and Dangl, 2006).

In wheat-rust pathosystems, R-genes mostly conform to Flor's gene-for-gene hypothesis. Gene-for-gene" model was first described by Flor (1955) in the pathosystem flax (*Linum usitatissimum*)-flax rust (*Melampsora lini*), postulates that for every 'R' gene in the host there is a corresponding avirulence (*Avr*) effector gene in the rust pathogen. The *R* gene encodes a protein, which recognizes the pathogen effector molecules through direct (gene-for-gene) or indirect (guard and decoy hypothesis) recognition and triggers the defense response. In the gene-for-gene model, the recognition between the *Avr* gene in the pathogen and the corresponding *R* gene in the host results in disease resistance (incompatibility) and elicits plant defense mechanisms, including hypersensitive reaction or localized cell death at and around the site of infection which limits further growth of the pathogen (Dangl et al. 1996). If either of the *R* gene or *Avr* gene is absent, no recognition occurs which results in disease susceptibility (compatibility). Gene-for-gene interactions has been reported in rice-*Magnaporthe oryzae* pathosystem, between rice blast resistance gene Pita product and the corresponding AvrPita gene product of rice blast fungus *M. oryzae* (Jia et al. 2000). In this interaction, the effector molecule AvrPita directly binds to the intracellular receptor, R protein Pita. This direct recognition and

interaction of the Avr and R protein trigger the induction of HR in rice plants which contributes to resistance. Another example is well characterized in between Flax- *M. lini* (Flax rust fungus) interaction (Dodds et al. 2006). Flax rust fungus gene products (AvrL5, L6 and L7) are directly recognized by flax resistance gene proteins L5, L6 and L7, which triggers the resistance defense response (Dodds et al. 2004).

The guard model postulates the indirect recognition of effector proteins by *R* gene, and triggers disease resistance in the host. The *R* gene monitors or guards the host target protein that is targeted by the effector protein. The recognition of the host target protein by effector proteins results in modification/phosphorylation of the target protein. This modification triggers the disease resistance in resistance plants (Dangl and Jones, 2001). However, guarded effector targets are polymorphic for resistance R genes and evolutionarily unstable in plant populations (Vander Hoorn et al. 2010). That means, the absence of functional R genes in the plant population decreases the binding affinity with the effector and can be modified/phosphorylated by the pathogen. In the case of presence of functional R gene in the plant population, then the binding affinity of guardee improves with the effector and enhances the pathogen recognition (Vander Hoorn et al. 2010). Guard model has been well characterized in Arabidopsis-*Pseudomonas syringae* interaction (Mackey et al. 2002). In this system, Arabidopsis, R-protein RPM1 confers resistance against *P. syringae* type III effector proteins AvrRpm1 and AvrB. RIN4 is a host-protein, which is required for normal growth, and development of Arabidopsis plants is the target of AvrRpm1 and AvrB proteins. RIN4 is also required for the induction of HR in RPM1-AvrRpm1 and AvrB interactions. AvrRpm1and AvrB upon interaction with RIN4 induce phosphorylation and result in the activation of RPM1-mediated HR. However, reduced levels of RIN4 protein induces HR and associated ion leakage in Arabidopsis, therefore, RIN4 is

considered as a negative regulator of defense response (Mackey et al. 2002 and Vander Hoorn et al. 2010). Therefore, RIN4 acts as the guardee and which is monitored by R proteins RPM1 (Kim et al. 2005).

The decoy model suggests, the indirect recognition of effector proteins by *R* gene. The host has proteins known as decoys, which are the targets of pathogen effectors (Vander Hoorn et al. 2008). Decoys evolution is independent of effector proteins and might have evolved from a gene duplication of the effector targets and acts as a structural mimic of the effector targets to recognize the pathogen. Unlike the guardee, decoy does not affect the binding affinity or interaction with the effectors in the absence of R protein (Vander hoorn et al. 2011). Decoy model has been supported by recent findings in *P. syringae*- Arabidopsis and Tomato interaction (Xiang et al. 2008). *P. syringae* effector AvrPto interacts directly with the FLS2 and EFR receptor kinases in Arabidopsis and LeFLS2 receptor kinases in tomato. In AvrPto - FLS2 interaction, AvrPto suppress the function of the FLS2 and blocks the PTI. But, not in the case of Arabidopsis fls2 mutants suggesting that AvrPto is required to suppress the PTI. In addition, AvrPto interacts with the host protein kinase Pto and triggers ETI in the tomato plants. A similar response was observed even in the absence of protein kinase Pto in the host. Suggesting that FLS2 and other receptor-like kinases are the targets of AvrPto and Pto might be the decoy which is independent of its evolution and does not involve in host resistance or susceptibility response (Xiang et al. 2008 and Vanderhoorn et al. 2011). Another example for decoy model is characterized in *Cladosporium fulvum*-Tomato interaction (Roony et al. 2005). In this study, the effector protein of *C. fulvum* fungus Avr2 interacts with the LRR-like Cf-2 protein of the host and triggers ETI including HR. But, this interaction also requires defense induced pathogenesis-related proteins RCR3 and PIP1 of tomato. Both these proteins accumulate in low and high

levels respectively, during the pathogen infection. However, the defense response occurred in the mutants rcr3-Avr2 is similar to the interaction between the tomato plants lacking Cf2 proteins. This suggests that PIP1 is the target of the effector protein Avr2 and triggers HR in which RCR3 acts as a decoy.

Host resistance to rust pathogens is conferred by resistance (R) genes designated as *Lr* (Leaf Rust), *Sr* (Stem Rust) and *Yr* (Yellow/stripe Rust). Till date, over 71 leaf rust resistance (*Lr*) genes have been characterized in wheat and its wild relatives (McIntosh et al. 2010 and Kolmer et al. 2013). *Lr* genes, *Lr1*, *Lr2a*, *Lr3*, *Lr10*, *Lr11* were characterized in hexaploid wheat, *Triticum aestivum* (Soliman et al. 1964). *Lr2a*, *Lr2b* and *Lr2c* were mapped in *Triticum aestivum* (hexaploid wheat) on the short arm of chromosome 2D (McIntosh and Baker, 1968). *Lr3a*, *Lr3g* and *Lr3ka* were mapped on the long arm of chromosome arm 6B (Haggag and Dyck, 1973). Genes *Lr14a* and *Lr14b* were mapped on chromosome 7BL which were tightly linked and are considered as alleles (Dyck and Samborski, 1970). *Lr17a* and *Lr17b* were mapped on 2AS locus (Dyck and Kerber, 1977). *Lr21* was later characterized from *T. tauschi* (Browder, 1980). *Lr22a*, *Lr22b* were mapped at a locus 2DS (Rowland and Kerber, 1974). Some of the *Lr* genes were introduced from wild species into common wheat; these are *Lr9* from *Aegilops umbellulata*, (Browder, 1980) *Lr26* from common rye, *Secale cereal* (Browder, 1980). Genes *Lr22a*, *Lr32*, *Lr39*, *Lr40*, *Lr41*, *Lr42* and *Lr43* were introgressed from *Aegilops squarrosa*. *Lr19*, *Lr24*, and *Lr29* from *Agropyron elongatum*, *Lr28*, *Lr35*, *Lr36*, *Lr47* from *Aegilops speltoides*, and *Lr37* from *Aegilops ventricosa* (Todorovska et al. 2009). *Lr1*, *Lr3a*, *Lr13*, *Lr17a*, and *Lr24* are common in Australian wheat lines (McIntosh, 1992). Also, many South American wheat cultivars might carry *Lr13* and *Lr34*. In European winter wheat, genes *Lr13*, *Lr3a* and *Lr26* are very common (Zadoks and Bouwman, 1985, Pathan and Park, 2006).

Host resistance can be sub divided into seedling or all-stage resistance, which is often race-specific and confers resistance through R-genes, and adult plant resistance, which is often non-race-specific and confers resistance through APR genes. However, some of the APR genes may be race-specific. For example, in wheat cultivar "Camp Remy" durable resistance to stripe rust, resistance genes are differently expressed at the adult plant stage as compared to the seedling plant stage (Collins et al., 2007; McIntosh, 2009).

Seedling plant (all-stage) resistance

Seedling resistance in the wheat-rust pathosystems is also known as race specific resistance or major gene resistance because this resistance is governed by a single major gene and is effective only against specific races/isolates of pathogen population. Seedling resistance genes confer resistance beginning at the seedling stage, and the resistance remains effective even in the adult-plant stage. Wheat leaf rust seedling resistance/race-specific resistance genes viz., *Lr1* (Cloutier et al. 2007), *Lr10* (Feuillet et al. 2003) and *Lr21* (Huang et al. 2003) have been cloned. All these resistance genes encode for nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins, which are one of the largest families of resistance genes in plants. Based on the structure of the *N*-terminus, NBS-LRR proteins are subdivided into two classes: *N*-terminal Toll-interleukin 1 receptor (TIR) domain (TIR-NBS-LRR), and putative coiled-coil domain (CC-NBS-LRR) (Gong et al. 2013). The NBS-LRR gene proteins in plants are involved in providing resistance against pathogens by direct/indirect interactions (Jones and Dangle, 2006). The NBS domains are involved in signaling and contain highly conserved motifs including P-loop, kinase-2 and Gly-Leu-Pro-Leu motifs (Marone et al. 2013). The LRR domains are structural domains, which involves in protein-protein interaction with pathogen effectors. LRRs are under diversifying selection and can evolve into different binding specificities (Marone et al. 2013).

This suggests that LRRs are under high selection pressure and aids in emerging virulent races of the pathogen.

Seedling resistance is characterized by the presence of HR and induction of rapid cell death at the site of infection in the epidermal and mesophyll cells (Rubiales and Niks 1992, Bolton et al. 2008). Seedling resistance in the host varies greatly from the slight reduction of fungal growth to strong HR, with reduced penetration and very little growth of the fungus (Xavier et al. 2001). Seedling resistance can be easily breakdown by the new virulent races of the pathogen resulting in the breakdown of the resistance in a short period of time. Since, the resistance with major genes effectiveness breaks down easily and do not provide long-term resistant, the race-specific resistance genes should be pyramided in wheat cultivars to develop stable sources of resistance (Leornard and Szabo, 2005) and the research has been mainly focuses on using adult plant/non-race specific resistance which remains durable.

Adult-plant resistance

Adult-plant resistance is defined as an incomplete form of resistance with some degree of spore production and uredinium formation (Dyck et al. 1966). APR resistance is best expressed in adult plant stage (Parlevliet, 1985) however, from recent studies, the expression can be induced at seedling stage under specific temperature and light combinations (Lagudah et al. 2011). Many APR genes confer a slow-rusting, non-race specific form of resistance which may remain durable. Non-race specific APR genes are of great value because broad-spectrum resistance can be obtained by a single gene (Navabi et al. 2005). APR provides resistance, by delaying the infection process and reducing the development of symptoms (Rubiales and Niks, 1995). Since the pathogen is able to grow and reproduce to some level (non-immunity, partial resistance) lower selection pressure is applied on pathogen compare to using HR-type of

resistance. However, some of the APR genes confer race specific resistance, for example, *Lr* genes including *Lr12, Lr13, Lr22b, Lr35, Lr37, Lr48* and *Lr49* confer race specific APR and some genes have been proven not durable which may belong to the class NB-LRR (Dyck and Kerber, 1985, Mc Callum et al. 2010 and Saini et al. 2002) and conform to Flor's gene-for-gene hypothesis (Flor, 1971). Non-race specific adult-plant resistance genes include *Lr34* (Dyck, 1987), *Lr46* (Singh et al. 1998) *Lr67* and *Lr68* (Hiebert et al. 2010). *Lr34*, *Lr46* and *Lr67* are associated with the expression of leaf tip necrosis (LTN) and also shown to have pleiotropic effects to multiple wheat pathogens including *Puccinnia graminis* f. sp *tritici* (wheat stem rust), *Puccinia triticina* (wheat leaf rust), *Puccinia striiformis* f. sp. *tritici* (wheat stripe rust) and *Blumeria graminis* f. sp. *tritici* (powdery mildew). Non-race specific and slow rusting genes such as *Lr34*, *Lr46* and *Lr67* are of great value because these genes offer resistance against a wide range of pathogen races contributing to durable resistance. Additionally, disease resistance to more than one disease can be obtained by a single gene.

Lr34/Yr18/Pm38/Ltn1 gene was the first identified by Dyck and Samborski, in 1979 and mapped to wheat chromosome 7D. *Lr34* was cloned from the wheat cultivar Chinese Spring and encodes a putative ABC transporter protein (Krattinger et al. 2009). The genomic sequence of *Lr34* contains 24 exons and spans 11,805 bp, which encodes a predicted protein of 1401 amino acids., *Lr34* is best expressed in adult-plant stage at 20°C but also can be expressed in seedling plant stage under cold temperatures. Thus, *Lr34* may be more effective in the regions where temperatures are lower during the growing seasons (Rubiales and Niks, 1995 and Singh et al. 2007). *Lr34* confers partial, non-race specific resistance to all tested isolates of wheat leaf and stripe rust pathogen species and hence is known to provide broad spectrum resistance. *Lr34* is attributed to early abortion of urediniospores germination and germ tube formation during

penetration (Rubiales and Niks, 1995). *Lr34* may also reduce the rate of haustoria formation and increases the latency period, the time period between initial infection and uredinium development (Bolton et al. 2008). The increased latency period helps keep inoculum at low levels in the growing season (Singh and Huerta-Espino, 2003). In addition to conferring resistance to leaf rust, it was shown that *Lr34* is same as *Yr18* which confers adult plant resistance to stripe rust (Singh et al. 1992), and powdery mildew resistance gene (*Pm38,* Lillemo et al. 2008) and also in association with expression of leaf tip necrosis (*Ltn1*). The LTN phenotype has been used as a phenotypic marker to select for APR. However, the severity of LTN is strongly influenced by environment (Navabi et al. 2005). The resistance provided by *Lr34* alone may not be sufficient under high infection pressure (Singh and Huerta-Espino, 1997). *Lr34* has been shown to work in an additive manner with other unknown APR genes; it has shown to enhance the effect of resistance genes, for example, combination of *Lr34* and *Lr16* can provide a greater level of resistance compared to the effect of individual gene (German and Kolmer, 1992).

Lr46/Yr29/Pm39/Ltn2 confers resistance to leaf rust, stripe rust, powdery mildew and exhibits leaf tip necrosis. *Lr46* was first described by Singh et al (1998) in spring wheat cultivar 'Pavon 76' and is considered as an APR, slow-rusting resistance gene. *Lr46* has remained effective in Mexico and other parts of the world since its release in 1976. The *Lr46* locus, was mapped to the terminal portion of the long arm of chromosome 1B through an analysis of substitution lines for the chromosomes of the resistant cultivar Pavon 76 backcrossed to susceptible cultivar Lalbahadur (Singh et al. 1998). William et al. (2003) used AFLP markers to map *Lr46* on the distal end of chromosome 1BL. *Lr46* do not provide complete immunity to host plant against a set of *P. triticina* races instead, delays the infection process caused by a wider

range of *P. triticina* races (Martinez et al. 2001). The effect of *Lr46* is similar to *Lr34* in exhibiting non-hypersensitive defense response to wheat leaf rust with prolonged latency period and causes a higher percentage of early abortion and in addition reduced the size of the colony (Martinez et al. 2001). *Lr46* also confers partial resistance to stripe rust (William et al. 2002). The severity of LTN associated with *Lr46* is lower than the leaf tip necrosis associated with *Lr34* (Rosewame et al. 2006).

Lr67/Yr46/Sr55/Pm46/Ltn3 contributes to partial resistance to leaf rust, stem rust (*Sr55*), stripe rust (*Yr46*), and powdery mildew (*Pm46*) and is also associated with leaf tip necrosis (*Ltn3)* (Sybil et al. 2011, Sybil et al. 2014 and Wolfgang et al. 2013). *Lr67* is derived from common wheat accession PI 250413 and was transferred into Thatcher to produce a backcross line RL6077. Wheat accession RL6077 is a Thatcher near-isogenic line that carries *Lr67/Yr46* on chromosome arm 4DL (Hiebert et al. 2010 and Herrera-Foessel et al. 2011). *Lr67* has been recently cloned and encodes a predicted hexose transporter (*Lr67*res) (Moore et al. 2015). *Lr67* resistance protein consists of 514 amino acids, which contain 12 predicted transmembrane helices (Moore et al. 2015). This is very similar to the STP (sugar transporter) 13 family of H^+/monosaccharide symporters. Genes that function as sugar transporters and facilitate the transport of sugars (glucose and sucrose) across the plasma membrane are as known as SWEET (Sugars Will Eventually be Exported Transporters) genes encodes transmembrane proteins (Xuan et al. 2013). Sugars are known to involve in multiple physiological processes including plant growth and development and also serves as a substrates and signals during plant defense responses (Lapin et al. 2013). During biotrophic interactions, the plant provides carbon and other nutrients in the form of sugars to the pathogens. Leaf tissues infected with pathogen show raised expression of SWEET sugar transporters, cell wall-bound vacuolar invertases and become a sink

for sugars (green islands) (Lapin et al. 2013). Green islands are known to surround the powdery mildew and rust infection sites, which maintain tissue photosynthesis during infection. There is evidence that activation of sucrose transporter-encoding SWEET genes plays a role in host susceptibility. For example, some of the SWEET genes have been transcriptionally induced in Arabidopsis and rice during bacterial and fungal infection (Pilot et al. 2013). In grapevine SWEET gene orthologs *VvHT1* and *VvHT5* (hexose transporters) have been transcriptionally activated after downy and powdery mildew infection. Another SWEET gene OsSWEET14 (rice sucrose/glucose transporter), transcription has been activated by a *Xanthomonas oryzae* pv. *oryzae* (Xoo) TAL effector, *AvrXa7* (Laptin et al. 2013). *Lr67*res allele differs from the *Lr67* susceptible (*Lr67*sus) allele form of the same protein by two non-synonymous single-nucleotide polymorphisms (SNPs) resulting in two amino acids, Arg144Gly and Leu387Val, substitutions conserved in orthologous hexose transporter (Moore et al. 2015). *Lr67*res utilizes a dominant-negative interference mechanism, on transporter activity mediated by forming inactive heterodimer protein complexes which reduce glucose uptake (Moore et al. 2015). The gene *Lr67* shares similar characteristics with *Lr34* in conferring partial or slow-rusting, non-race specific or broad spectrum adult plant resistance to leaf rust and stem rust and also is associated with enhanced stem rust resistance in thatcher (Wolfgang et al. 2013). *Lr67* is closely linked or pleiotropic to stripe rust resistance gene *Yr46*.

Resistance mechanisms to rusts

Numerous studies have been conducted to understand the different types of resistance mechanisms involved in rust pathogen-host interactions. Histological studies on rust-host interactions, revealed mainly two types of resistance mechanisms. Rust induced resistance was categorized into pre-haustorial and post-haustorial resistance mechanisms based on the ability of

the pathogen to form to haustoria in the host plants by a rust fungus. These resistance mechanisms have been studied in both host and non-host- rust pathogen interactions. Understanding different types of resistance mechanisms play a key role to achieve durable resistance. The combination of different resistant mechanisms would enhance the effect of resistance and achieve durability.

Pre-haustorial resistance

The pre-haustorial resistance is also known as pre-cell wall penetration of host resistance mechanism since the resistance is expressed before the penetration of the fungus into the host. Pre-haustorial resistance mechanism was first described by Heath (1981) in rust fungus and non-host interactions as reduced penetration of fungus into host tissue. Later Niks (1982) described the pre-haustorial resistance mechanism in detail in barley-leaf rust interactions. In this study, partially resistant barley seedlings were infected with leaf rust fungus *Puccinia hordei* and leaf samples were evaluated at early time points. The pre-haustorial mechanism in this study was described as the failure of haustorium formation by rust fungus on barley leaves. Failure of haustorium formation leads to the slower development of rust fungus (longer latency period) and lower or sometimes non-production of spores (no sporulation). A local apposition of cell wall material known as papilla is observed near the penetration site. This papilla may not only involve in blocking the pathogen, but it may also be involved in cell wall repair after the penetration attempt by the pathogen (Nicks and Rubiales, 2002 and Collins et al. 2007). Echevarria et al. (2006) when conducting histological studies in sunflower- broom rape (*H. annuus– O. cumana*) interaction, revealed that pre-haustorial resistance prevented pathogen penetration by suberization and protein cross-linkage in the cell walls of resistant sunflower plants. Fluorescence and confocal laser microscopy observations also revealed the accumulation of

phenolic compounds during resistance reactions (Echevarria et al. 2006). Similarly, pre-haustorial resistance mechanisms were observed in *Tc-Lr9* infected with wheat leaf rust pathogen *P. triticina* (Wang et al. 2013). This study revealed that pre-haustorial resistance mechanism involved in the deposition of callose and cellular lignification around the site of pathogen penetration. In addition, induction of an HR which is associated with cell death in the neighboring infected cells was observed (Wang et al. 2013).

Pre-haustorial resistance is most commonly observed in non-host interactions where there is no basic compatibility exists (Heath, 1997). Therefore, it would be considered as the basal first defense mechanism of the plant to be encountered by the pathogen. If the pre-haustorial mechanism is not sufficiently strong or quickly expressed, the pathogen will be able to enter the plant cell and form infection structures including SSV, in the stomatal cavity of the host and enter host intercellular spaces and form infection hyphae and HMCs and even sometimes haustoria then which a post-haustorial mechanism may be elicited (Jacobs, 1989).

Post-haustorial resistance

Post-haustorial resistance can also be known as post-cell wall penetration resistance. In this type of resistance, the fungus is able to penetrate the host cells and form haustorium by invaginating the host mesophyll cells (Niks and Dekens 1991). After the formation of at least one haustorium or sometimes, after the establishment of some colonies by the fungus, post-haustorial resistance can be induced (Orczyk et al. 2010). In this type of resistance, the plant cell with haustoria usually dies and blocks the pathogen growth and development in the neighboring cells (Silva et al. 2002). This defense reaction of the host to the pathogen attack is called HR (Niks and Dekens, 1991). In Post-haustorial resistance, HR is induced immediately after the formation of a haustorium in the host cells. Post-haustorial resistance is more commonly observed in host-

pathogen interactions where the host carries race specific resistance (Heath et al. 1982). Post-haustorial resistance and cell death were characterized in the coffee orange rust fungus *Hemileia vastatrix*-coffee leaves interaction (Silva et al. 2002). In this study, inhibition of fungal growth was observed in haustoria-invaded mesophyll cells in the resistant coffee leaves. Reduction in hyphal length and number of haustoria per infection site was observed after the formation of one haustorium (two days after inoculation) in the plant cell. Haustorial encasement with callose and β-1, 4 glucans and hypertrophy of the host cells was also observed (Silva et al. 2002).

In post-haustorial resistance mechanism, a localized HR was observed upon the attack of the pathogen, which in turn is characterized by the rapid death of the plant tissue (necrosis/HR) at the site of infection (Lam, 2004). This HR involves in the production of pathogenesis-related (PR) proteins, secondary metabolites synthesis and cell wall reinforcements (Dangl and Jones, 2001). HR is sometimes accompanied by the production of an oxidative burst at the plasma membrane which includes hydrogen peroxide, super oxides and hydroxyl radicals (H_2O_2, O_2^- and HO respectively) (Houot et al. 2001). This leads to the increased influx of Ca^{2+} levels, activated protein kinase cascades, transcriptional reprogramming (Lam et al. 2004). Among the ROS, H_2O_2 plays a key role in the defense mechanism of the plant. In plant cells, H_2O_2 accumulation acts in a dose-dependent manner. For example, lower levels of H_2O_2 is seen in plant cells during abiotic stress and high levels of H_2O_2 is seen during biotic stress in the plant cells. Higher levels of H_2O_2 accumulation leads to signaling pathway which in turn leads to the rapid programmed cell death (PCD). This leads to the increased influx of Ca^{2+} levels, activated protein kinase cascades, transcriptional reprogramming (Lam et al. 2004). Among the ROS, H_2O_2 plays an important role in the defense mechanism of the plant. H_2O_2 accumulation in the plant cells acts in dose-dependent manner. For example, lower levels of H_2O_2 accumulation is seen in plant cells

during abiotic stress and during biotic stress, higher levels of H_2O_2 accumulation occurs in the plant cells. Higher levels of H_2O_2 accumulation leads to signaling pathway which in turn leads to the rapid programmed cell death (PCD). The typical biochemical, morphological changes in PCD in plants includes shrinkage of cytoplasm, nuclear DNA cleavage, chromatin condensation and aggregation (Bozhkov et al. 2011). In addition, sometimes generation of apoplastic peroxidases and membrane-localized NADPH oxidases also appear in PCD (Bindschedler et al., 2006; Torres et al., 2002). These enzymes produce H_2O_2 which is required for triggering PCD in infected and surrounding infected cells (Torres et al., 2005). Similar morphological changes, including condensation, cytoplasm shrinkage, DNA fragmentation and apoptotic-like bodies formation are observed between animal cells undergoing apoptosis and dead plant cells (Wang et al. 1996, and de Jong et al. 2000, Lam et al. 2004, Pennell and Lamb, 1997). Additionally, ROS and HR-associated cell death is also induced by some phytohormones such as salicylic acid (SA) and ethylene (ET) (Gadjev et al. 2008). SA stimulates and/or suppresses the PCD which contributes to the establishment of border line between the living tissue and development of lesion depending on the concentration (Alvarez, 2000).

Post-haustorial resistance has been studied in both host and non-host interactions. In the rust-host interactions involving seedling/race-specific genes gene-for-gene resistance, post-haustorial resistance is exhibited by recognizing the pathogen in a direct/indirect manner as explained in seedling resistance section. In the non-host interactions, post-haustorial resistance might be exhibited at the site of haustoria formation and become encased in callose and the host cell undergoes the HR (Neu et al. 2003). Non-host post-haustorial resistance study on Brachypodium inbred lines inoculated with *P. graminis* f.sp. *tritici*, *P. graminis* f.sp. *lolii* and *P. graminis* f.sp. *phlei-pratensis* demonstrated that post-haustorial resistance was exhibited after the

formation of haustorium 68 hpi. By 8 dpi, dark flecks and necrosis were observed around the pustule development with all the three *P. graminis* isolates (Figueroa et al. 2013).

The major difference between the gene-for-gene post-haustorial resistance and non-host post-haustorial resistance is that the former occurs mainly after haustorium formation, whereas the later occurs beginning after the penetration of the pathogen and continues to exhibit resistance after the haustoria formation and mostly is associated with the HR (Cheng et al. 2012). However, the HR is a common feature observed with the low frequency of non-host penetrations where initiation of haustoria occurs (Neu et al. 2003).

Objective

The goal of this work is to identify physical and molecular components associated with resistance mechanisms to the wheat leaf rust pathogen *Puccinia triticina*.

Specific objectives

[1] Histological evaluation of pre-and post haustorial resistance mechanisms to leaf rust pathogen *Puccinia triticina* in wheat and barley.

a. Investigate physical and biochemical events occurring during the infection process of *P. triticina* on near-isogenic wheat genotypes and barley lines carrying different *Lr* genes at adult and seedling plant stage.
b. Investigate and compare the molecular basis of race-specific, non-race-specific, and non-host resistance of wheat and barley to *P. triticina.*

[2] Develop a quantitative PCR assay for detection and quantification of fungal biomass in wheat and barley infected by *P. triticina* and determine its potential used for resistance mechanism characterization.

[3] Quantify the expression of specific genes during early stages of infection in wheat NILs carrying Tc-*Lr9* and Tc-*Lr21* genes.

[4] Development of a rapid and efficient protocol for better evaluation of all infection structures using high-resolution epifluorescence microscopy.

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CHAPTER TWO: HISTOLOGICAL EVALUATION OF PRE-AND POST HAUSTORIAL RESISTANCE MECHANISMS TO THE LEAF RUST PATHOGEN *PUCCINIA TRITICINA* IN WHEAT AND BARLEY.

Abstract

Achiving durable resistance to diseases caused by hyper-variable plant pathogens such as P*uccinia triticina,* has proven to be challenging. The infection process of *P. triticina* in wheat and barley genotypes was studied by fluorescence microscopy to better understand the physical mechanisms involved in resistant reactions caused by different resistance genes. Wheat cultivar 'Thatcher' near isogenic lines carrying different leaf rust resistance genes (*Lr*) were infected with races of *P. triticina* to understand how different genes affect pathogen colonization in adult and seedling plant stages in compatible and incompatible interactions. In addition, resistance mechanisms involved in the host (wheat) and near- non-host (barley) genotypes, were investigated. Wheat line Tc-*Lr9* and barley line Q21861 were categorized as carrying pre-haustorial resistance since inhibition of pathogen growth was observed after appresorium formation and no haustorial mother cells or haustoria were produced in incompatible interactions. Thatcher near-isogenic lines, Tc-*Lr34,* and Tc-*Lr35* were characterized as carrying post-haustorial resistance as evidence of incompatibility was observed after haustoria were produced. In the lines carrying pre-*haustorial* resistance (Tc-*Lr9* and barley line Q21861), a localized hypersensitive response (HR) and subsequent cell death were observed at the site of appresorium formation (6 hpi). Tc-*Lr21* was characterized as carrying pre-haustorial and post-haustorial resistance as inhibition of pathogen growth was observed before penetration of the pathogen and after haustoria formation. In total, four types of resistance mechanisms including pre-haustorial resistance with HR, pre-haustorial resistance without HR, post haustorial

resistance without HR and post haustorial resistance with HR were observed in *Lr* genes carrying race-specific resistance.

Introduction

Wheat (*Triticum aestivum* L.) is the world's leading cereal grain and one of the most important food crops grown worldwide. Leaf rust is the most important foliar disease of wheat worldwide (Kolmer et al. 2009). Leaf rust occurs wherever wheat is grown and it is most widely distributed of all cereal rusts causing yield losses and significantly reducing seed quality (Bolton et al. 2008). Yield losses and disease severity depend upon the time of infection and susceptibility levels in the cultivars. Losses due to leaf rust are usually small (< 10%), but can be 30% or more in the case of severe infections (McMullen et al. 2008). Use of fungicides is an effective measure to manage rust diseases but it requires specific application timing (prior to infection), which is not always possible (Markell et al. 2006). Use of resistant varieties is the primary means of rust disease management strategy and is more economical (Martinez et al. 2001). To date, 71 wheat leaf rust resistance (*Lr*) genes have been designated (Kolmer et al. 2013) but only a few are effective against the highly diverse North American *P. triticina* population since most of these genes show race-specific resistance. Race-specific resistance is mostly governed by single resistance genes, involving hypersensitive reaction (HR). Fungal biotrophic pathogens can easily defeat the cultivars carrying race specific resistance (Anker and Niks, 2001). Another type of resistance is the non-race specific resistance, partial resistance, which is commonly governed by multiple genes (Singh et al. 2011). This type of resistance shows an increase in latency period and decreased infection frequency and uredium size (Rubiales and Niks, 1995). The identification, characterization and utilization of broad, non-host resistance and/or partial resistance, which is non-race specific, is an attractive strategy for the

development of effective and possibly more durable leaf rust resistant wheat varieties. Histopathological studies provide valuable information to study the infection process involved in the host-parasite interaction. Previous histological examinations of wheat-rust interaction have revealed pre- and post-haustorial resistance components (Niks & Dekens, 1991).

Pre-haustorial resistance is a part of plant's general defense mechanism. During this resistance mechanism, the rust pathogen is able to form infection structures on the leaf surface and can sometimes penetrate into the mesophyll cells. Pre-haustorial resistance has been described as, a reduced penetration of fungus into host tissue and as the failure of haustorium formation by rust fungus (Heath, 1981 and Niks, 1982). In this mechanism, the urediniospores develop normal haustorial mother cells after penetration but further development of haustoria is arrested due to callose deposition on the cell wall or near the penetration site which leads to longer latency period and no sporulation. This local apposition of cell wall material, is known as papilla and it is involved in blocking the pathogen (Nicks and Rubiales, 2002 and Collins et al. 2007). *H. annuus–O. cumana* incompatible interaction involves suberization and protein cross-linkage in the cell walls of resistant sunflower plants, preventing the pathogen penetration (Echevarria et al. 2006). Pre-haustorial resistance is most commonly observed in non-host interactions where no basic compatibility exists (Heath, 1997). Pre-haustorial resistance would be considered as the first defense mechanism of the plant to be encountered by the pathogen. If the pre-haustorial mechanism is not sufficiently strong or quickly expressed, the infection unit will be able to enter the plant cell and form infection structures including SSV in the stomatal cavity of the host and enter into the host intercellular spaces, developing infection hyphae and HMCs and even sometimes haustoria before post-haustorial mechanism is elicited (Jacobs, 1989). The biochemical changes and cellular compounds might contribute to resistance but, the

molecular machinery underlying them, is not well known (Collins et al. 2007, Mallard et al. 2008 and Tuzun, 2001). The relationship between the resistance mechanisms and potentially durable resistance (race-non-specific, non-host, and pre-haustorial) is still unanswered. Understanding the different resistance mechanisms underlying host-parasite interactions and steps involved in the infection process of pathogenesis to leaf rust is key for developing wheat varieties with durable resistance. In the present study, Ttiming of pathogen infection structures development and plant response was evaluated using fluorescence microscopy. The infection process of *P. triticina* at two growth stages (seedling stage and adult-plant stage) and the relationship among the resistance mechanisms involved in different *Lr* genes were investigated in the first set of experiments. In the second set of experiments, race specific and race-non-specific resistance mechanisms at seedling plant stage using two different races *P. triticina* races, THBL and MCDL in a time course experiment was investigated.

Materials and Methods

Plant material

Infection process of wheat leaf rust pathogen *P. triticina*, was characterized in wheat and barley. NILs developed by P.L Dyck and R.G Anderson of the Agriculture and Agri-Food Canada Cereal Research Centre in Winnipeg (Hayes et al.1936) were used. Wheat susceptible cv. Thatcher and its near isogenic lines (NIL'S) Tc-*Lr9,* Tc-*Lr21,* Tc-*Lr34,* Tc-*Lr35,* and barley lines Q21861 and Harrington were used for histopathological studies. These lines were selected for the evaluation of infection process, at seedling and adult-plant stage in the first experiment. To examine different resistance mechanisms and components involved in race specific vs. race non-specific resistance genes, Tc-NIL'S carrying race specific (Tc-*Lr9,* Tc-*Lr21,* Tc-*Lr35)* and race non-specific resistance genes (Tc-*Lr34*), were selected in the second experiment.

Additionally, diploid wheat relative species (*Triticum monococcum L.* subsp. *monococcum*) and barley (*Hordeum vulgare* L. subsp. *vulgare*) genotypes (Table. 1) were screened for pre-and post-haustorial resistance. Genotype material was obtained from the USDA-ARS National Small Grain Collection (NSGC).

Experimental design

All the experiments were conducted at the North Dakota State University Agricultural Experiment Station Greenhouse Complex (NDAES) in Fargo, ND. The experiment was arranged in a completely randomized design with three replicates and the entire experiment was repeated twice for each race of the pathogen. For each genotype, three seeds planted per cell were considered as an experimental unit. Genotypes were planted in a 24-cell trays with Sunshine® Mx #1 (Sungro Horticulture Distribution Inc., Quincy, MI, USA) with slow release seedling fertilizer (Osmocote 15-9-12, N-P-K, Everris NA Inc., OH, USA). Susceptible check Thatcher was included 3 times in each tray in all experiments.

Inoculations

Seedling plants were grown in trays in the rust-free greenhouse until second leaf stage was reached (primary leaf fully expanded approximately 7-8-day-old). Adult-plants were grown until flag leaves were fully expanded, approximately 45-50-days after planting. All experiments were conducted in greenhouses at 20-21^{o}C during day time and 16-18 ^{o}C during the night; daylight was supplemented for a photoperiod of 16h. Single spore isolates of *P. triticina* races THBL and MCDL were used for inoculations (Long & Kolmer, 1989). Inoculum consisted of fresh urediniospores at a concentration of 6.2 x 10^5 spores per ml in Soltrol 170 oil (Phillips Petroleum, Bartlesville, OK, USA). Inoculations were done using a spray inoculator. Mock inoculations were performed by spraying plants with Soltrol oil without urediniospores. After

inoculation plants were incubated in a mist chamber at 100% relative humidity for 16h in darkness and then transferred to a greenhouse compartment. Mock-inoculated plants were placed in a rust-free greenhouse compartment under the same temperature and light regime.

Time course sample collection and disease infection (IT) type rating

Samples were collected for visualization of fungal infection structures development under fluorescence microscopy. Non-inoculated leaf samples were collected before inoculation. Mock-inoculated leaf samples were collected immediately after spray with Soltrol 170 oil. Inoculated leaf samples were collected at 0 min, 6 hpi, 12 hpi, 24 hpi, 48 hpi, 72 hpi, 96 hpi, and 7 dpi for histopathological observations. Disease phenotype, based on infection type (IT), was recorded 14 dpi following the 0-4 rating scale where infection type 0 is considered immune (no visible sign or symptoms on plant) and infection type 4 corresponds to large uredia which aremay be surrounded by chlorosis (Stakman et al. 1962, Long D.L and Kolmer J.A, 1989). IT 0 to 2 are considered as avirulent reaction (host resistant response), while 3 and 4 were considered as a virulent reaction (host susceptible response).

Sample preparation for microscopic observations

Two three cm-long leaf pieces from the central portion of primary leaf for each genotype were collected and pooled for histological observations (Figure 1a). Leaf samples were placed in 20 ml disposable scintillation vials filled with leaf clearing solution (chloroform: methanol 3:1 v/v) and placed on ice bath and shaken for 5-6 hours or until the leaves were fully cleared (Figure 1b) on an orbital shaker (BioExpress S-3200-Ls Orbital Shaker Variable 115VAC, 60Hz, 0.24amps).

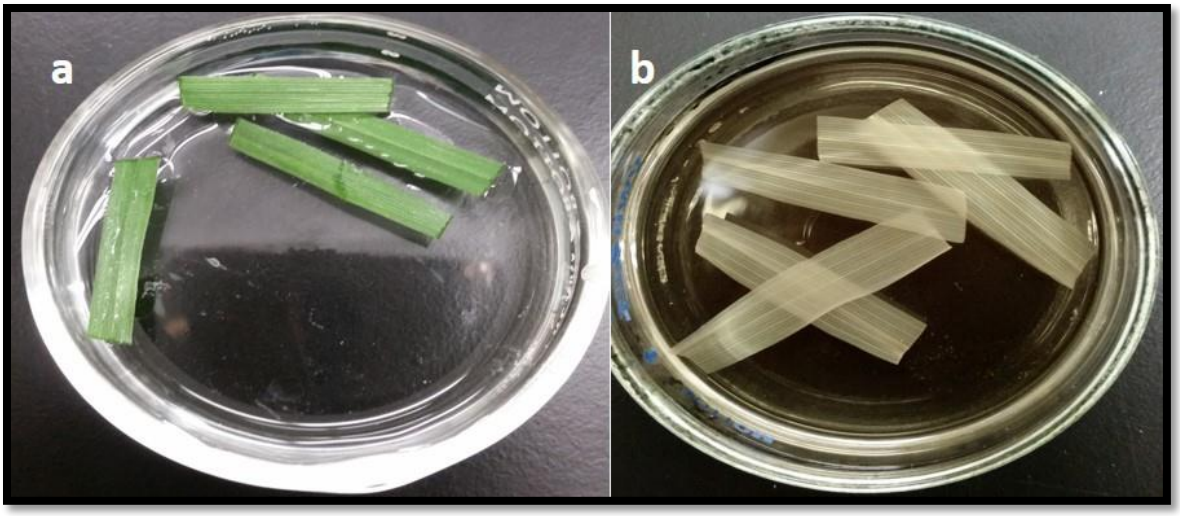

Figure 1. Clearing of wheat seedling leaf samples for histopathology study (a-b).

a) Leaf samples were collected and placed directly into chloroform: methanol 3:1 v/v immediately after collection for 5-6 hours. **b)** Samples were cleared in a clearing Solution after 5-6 hours (cleared leaf samples).

Uvitex 2B staining

The leaf segments were processed for fluorescence microscopy using modified method of Uvitex 2B staining procedure (Kuck et al. 1981). In short, after clearing, leaves were fixed by placing at 65^0C for 2 min in Lacto phenol/ethanol [1:2 v/v] and stored overnight in this mixture at room temperature. After washing for 15 min with 50% ethanol, twice for 15 min each with 0.005M NaOH, three times with water for 15 min., samples were placed in 0.1 M Tris /HCL buffer, pH 8.5 for 30 min. after, samples stained using 0.3% fluorochrome Uvitex 2B for 5 min. This was followed by washing four times with water [10 min each] and once with 25% aqueous glycerol [30 min]. Samples were mounted in glycerol containing a trace (10µl in 100ml) of lactophenol as a preservative and stored at 5˚C and in dark conditions. Samples were examined using a Zeiss Axio Imager M2 research epifluorescence upright microscope with an excitation filter BP379-401; chromatic beam splitter FT 420; emission 435-485; using AxioVision rel. 4.8 software using 5X, 20X, 40X lens.

Ten infection units were observed per each slide per time point per genotype and images of representative infection sites were acquired. Infection units were categorized as aborted if no fungal growth was observed after appresorium formation. Infection units with all the infection structures including haustoria were categorized as established colonies. The genotypes showing a majority of aborted colonies before haustoria was formed (pathogen arrested after appresorium formation) were considered as genotypes carrying pre-haustorial resistance. Genotypes exhibiting all infection structures including at least one haustorium and resistant IT were categorized as genotypes carrying post- haustorial resistance (Niks et al. 1991).

Colony size measurements

Colony size (area) was measured to compare pathogen growth in resistant and susceptible genotypes. Wheat seedling plants infected with *P. triticina* races MCDL and THBL and samples collected at 7 dpi were used for colony size analysis using Image Pro-plus software (Media Cybernetics, Inc.). Images acquired from fluorescent microscopy at 5X magnification were used to measure the colony size. Ten, randomly selected, colonies per leaf sample were measured. Area of each colony was measured by drawing a line along the circumference of the colony (Figure 2). Colony size data of *P. triticina* was analyzed using proc ANOVA. Mean separation of treatments was done by Fisher's Least Significant Differences Test (LSD). Genotypes were considered significant when p-values were significant by the t-test ($p=0.05$). All analysis was performed using SAS statistical package v 9.3.

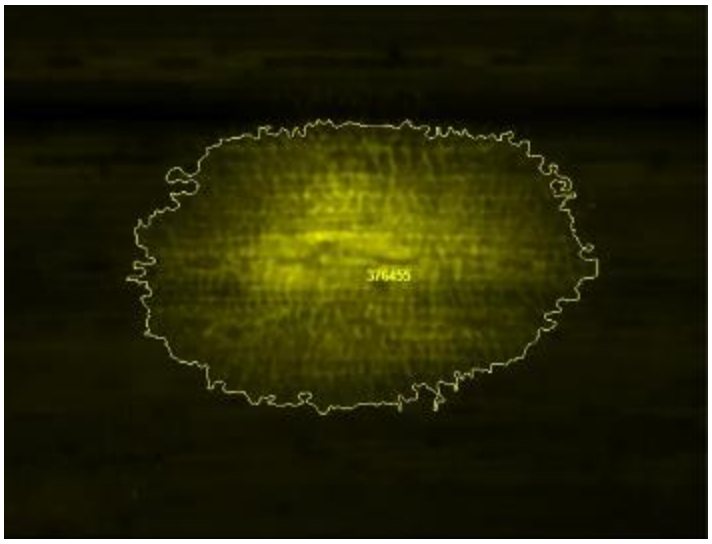

Figure 2. Image showing the colony area measurements using established colony images taken from fluorescence microscope and Image Pro-plus software.

Results

Microscopic observations

Infection structure development and disease progress of *P. triticina* in Tc-NILs and barley lines at seedling and adult plant stages were studied using fluorescence microscopy.

Seedling plant stage experiments inoculated with THBL and MCDL races: Susceptible cv. Thatcher was inoculated with *P. triticina* urediniospores of races THBL and MCDL. Pathogen growth and development was studied using fluorescence microscopy. Germination of urediniospores, branching of germ tubes and appresorium was observed within 6h after incubation (Figure 3a). Germinating urediniospores in many cases had 2-3 germ tubes but only one of the germ tubes differentiated into an appresorium with 3-4 lobes. Appressoria with lobes were observed in the stomata of leaves by 6 hpi (Figure 3b). After the appresorium was formed, other infection structures including infection peg and sub-stomatal vesicle, was observed by 12 hpi (Figure 3c). Infection hyphae and HMC were observed by 18 hpi (Figure 3d). Established colonies of the pathogen were observed by 24 hpi. In later time points, including 48 hpi, 72 hpi

and 96 hpi, further growth and development of the colony with newly formed spores was observed (Figure 3e). By 7dpi colonies with sporulation (matured and newly formed spores) were observed (Figure 3f). Similar temporal development of infection structures was observed with both races.

Adult-plant stage experiments inoculated with *P. triticina* race THBL: Flag leaves of Thatcher were infected with *P. triticina* race THBL. Infection structures development of the pathogen in the host were studied using fluorescence microscopy. Same temporal development of the pathogen as in seedling experiments was observed on the flag leaves.

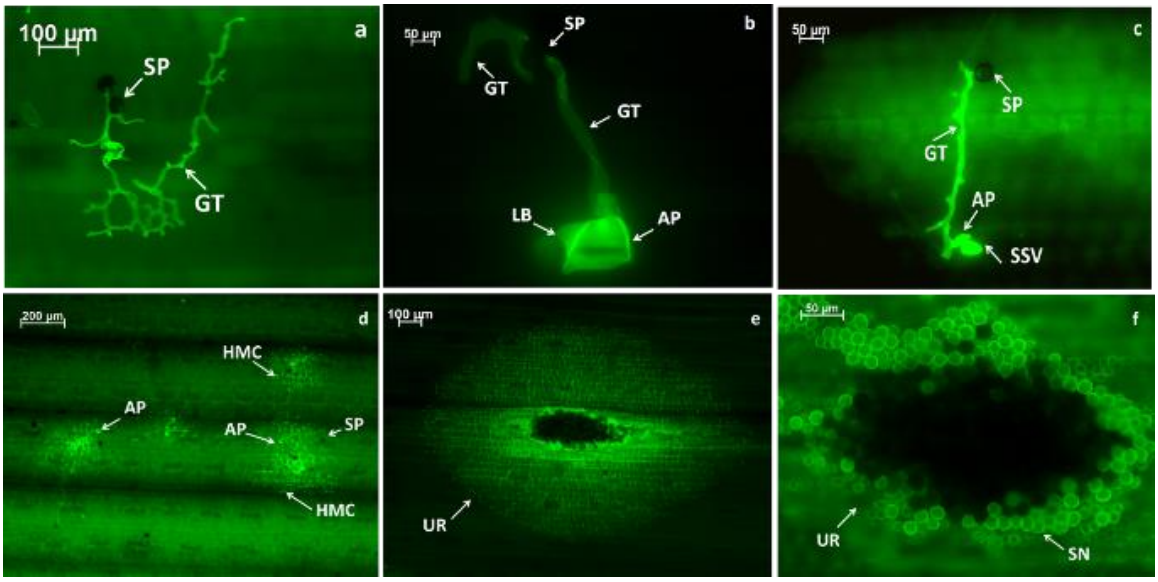

Figure 3. Infection structure development of wheat leaf rust pathogen *P. triticina* on susceptible wheat cv. Thatcher at seedling and adult-plant stages.

Plants were inoculated with *P. triticina* races THBL and MCDL and examined under an Axio Imager M2 Zeiss Research epifluorescence microscope. Representative images of fluorescence microscopy are shown (**a-f**).

a) Spore (SP). **b)** Branching germ tube (GT) and appresorium (AP) with lobes (LB). **c)** Infection peg formed from appresorium and subsequent sub-stomatal vesicle (SSV) formation from infection peg. **d)** Haustorial mother cell (HMC) produced a penetration peg invaginates through mesophyll cell wall to form a haustorium. **e-f)** haustorial mother cells, and uredinium development (UR) with sporulation (SP).

Seedling plant stage experiments inoculated with THBL and MCDL races: Tc-*Lr9* seedling plants incompatible reaction to *P. triticina* races THBL and MCDL was characterized. Spore germination (Figure 4a) and appresorium with 3-4 lobes were observed by 6 hpi (Figure 4b). After appresorium formation, no further growth or development of pathogen infection structures was observed. In addition, necrosis was observed in the cells next to and around the appresorium. Necrotic cells were clearly visible under a fluorescence microscope as very bright, highly stained cells (Figure 4c). Similar temporal development of the fungus was observed with MCDL race except that in few cases (3% of infection units), HMC were observed in the mesophyll cell wall while no haustoria were observed with THBL race. Abortion of colonies after appresorium formation was observed. No colony development or sporulation was observed by 7dpi.

Adult plant stage experiments with *P. triticina* race THBL: Pathogen infection structures development and disease progress were similar in the flag leaves of Tc-*Lr9* as seen in seedling leaves until appresorium formation (by 6 hpi). After appresorium formation, no further growth or development of infection structures and necrosis was observed in infection units in flag leaves.

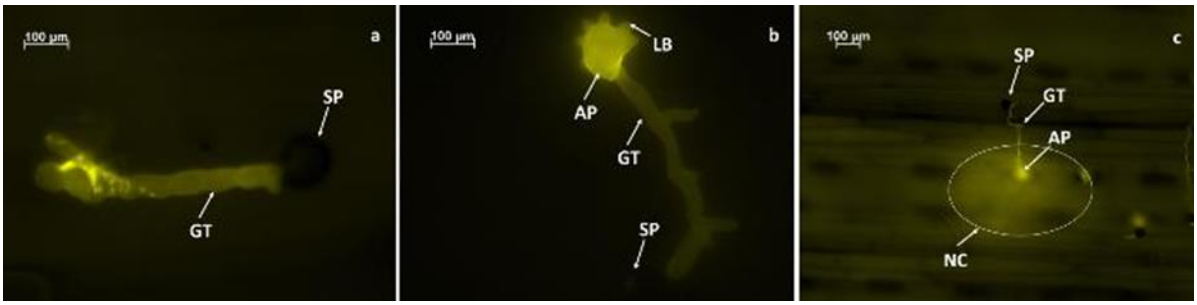

Figure 4. Infection structure development of *P. triticina* on resistant wheat Tc-*Lr9* at seedling and adult plant stages.

Plants were inoculated with *P. triticina* races THBL and MCDL and examined under an Axio Imager M2 Zeiss Research epifluorescence microscope. Representative images of fluorescence microscopy have shown (**a-c**).

a) Spore (SP) with germ tube (GT) at 2 hpi. **b**) Appresorium (AP) with lobes (LB) at 6 hpi. **c)** Necrosis (NC) at the site of appresorium (at 6 hpi) observed as bright and heavily stained cells.

Seedling plant stage experiments inoculated with *P. triticina* race THBL and MCDL: In the case of Tc-*Lr21*, incompatible interactions resulted in urediniospores germination and appresorium by 6hpi (Figure 5a). Other infection structures including infection peg and SSV, were observed by 12 hpi (Figure 5b). Infection hyphae and HMC were observed by 18 hpi (Figure 5c). Increase in the fungal growth and size of the colony in the 48 hpi, 72 hpi and 96 hpi was observed (Figure 5d). Uredinia formation and sporulation was observed by 7 dpi with each race (Figure 5e). Aborted colonies which were associated with necrosis was observed under a fluorescence microscope with race THBL only at 24 hpi and 48 hpi (AC, Figure 5g). In addition, sporelings with thin, long and branching germ tubes were observed at 48 hpi with THBL race only (Figure 5f).

Adult plant stage experiments inoculated with *P. triticina* race THBL: Abortion of some of the infection units was observed after 24 hpi. Aborted colonies were associated with necrosis, which was under a fluorescence microscope in the leaves of adult-plant stages (Figure 5h). However, smaller sized colonies were observed on Tc-*Lr21* than on Thatcher (Table 5).

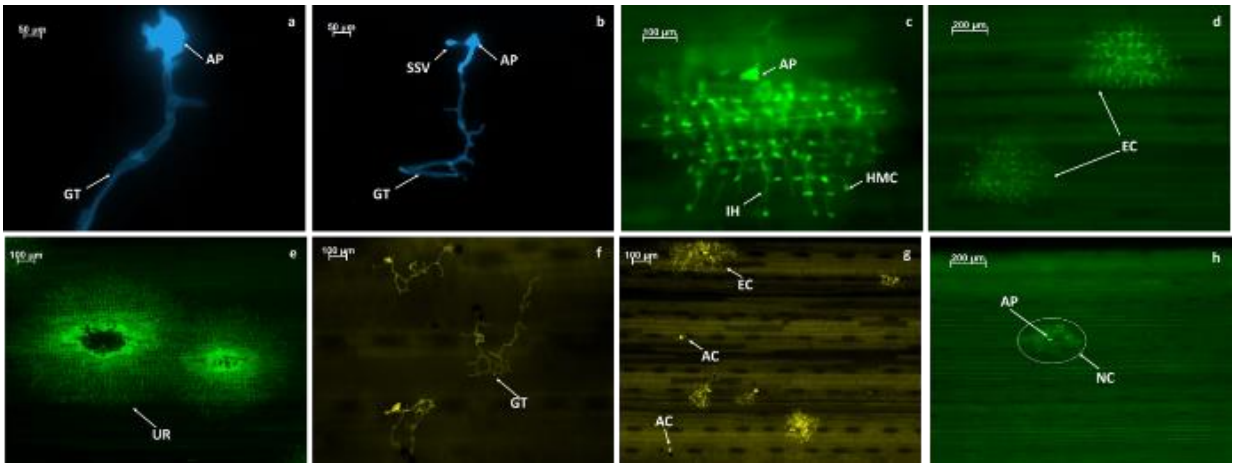

Figure 5. Infection structure development of wheat leaf rust pathogen *P. triticina* on Tc-*Lr21* at seedling and adult-plant stages.

Plants were inoculated with *P. triticina* races THBL and MCDL and examined under an Axio Imager M2 Zeiss Research epifluorescence microscope. Representative images of fluorescence microscopy have shown (a-h).

a) Branching germ tube (GT) and appresorium (AP) with lobes (LB) at 4 hpi. **b)** Infection peg formed from appresorium and sub-stomatal vesicle (SSV) at 12 hpi. **c)** Formation of haustorial mother cell (HMC) with penetration peg that invaginates mesophyll cell wall to formed a haustorium at 24 hpi. **d)** The increase in a number of haustorial mother cells and colony development. **e)** Uredinia formation with sporulation (SP) at 7dpi. **f)** An aborted colony with sporelings. Sporelings show thin and long germ tubes (GT) at 48 hpi. **g)** Early aborted colonies (AC) and established colonies (EC) at 48 hpi. **h)** Necrosis (NC) observed as bright and heavily stained cells at the site of aborted colonies at both time points 24 hpi and 48 hpi.

Seedling plant stage experiments with *P. triticina* race THBL and MCDL: On Tc- *Lr34* and Tc-*Lr35*, urediniospores germination, germ tube, appresorium formation was observed by 6 hpi and SSV was observed by 12 hpi (Figure 6a) when inoculated with race THBL and MCDL. Further growth and development of colony including SSV, infection hyphae and HMCs were observed by 24 hpi (Figure 6b). Complete colony development in the later time points including 24 hpi, 48 hpi, 72 hpi and 96 hpi and sporulation was observed by 7dpi similar to susceptible cv. Thatcher (Figure 6c). No abortion of infection units and no necrosis was observed with each race.

Adult plant stage experiments with *P. triticina* race THBL: In Tc-*Lr34* flag leaves, similar growth and development of the pathogen were observed as seen in seedling leaves until 24 hpi. However, after 24 hpi abortion of sporelings was observed and no further growth of the pathogen was observed (Figure 6c). No necrosis was associated with the cells around the aborted colonies (Figure 6d).

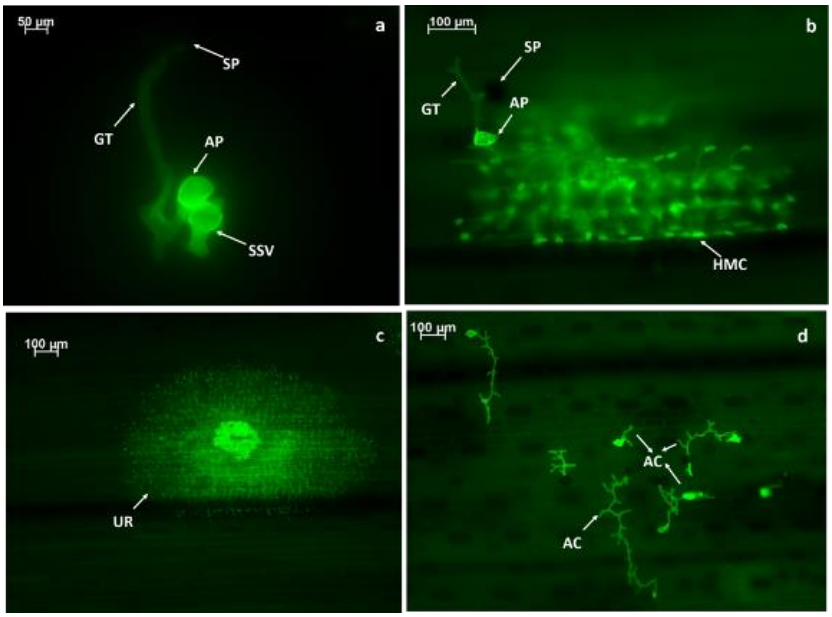

Figure 6. Infection structure development of *P. triticina* on Tc-*Lr34* and Tc-*Lr35* at seedling and adult-plant stages.

Plants were inoculated with *P. triticina* races THBL and MCDL and examined under an Axio Imager M2 Zeiss Research epifluorescence microscope. Representative images of fluorescence microscopy have shown (a-d).

a) Germ tube (GT) and appresorium (AP) with lobes (LB) and infection peg formed from appresorium and sub-stomatal vesicle (SSV) at 12 hpi. **b)** Formation from infection hyphae and Haustorial mother cell (HMC) produced a penetration peg that perforated through mesophyll cell wall and formed a haustorium in the host cell. **c)** Increase number of haustorial mother cells, uredinium formation (PL) at 48 hpi. d) An aborted colony with sporelings having thin and long germtube (GT) and appresorium (AP) at 24 hpi.

Seedling plant stage experiments inoculated with *P. triticina* race THBL and MCDL: Barley line Q21861, when challenged with each THBL and MCDL, sporelings germination, germtube and appresorium formation was observed by 6 hpi (Figure 7a). Early abortion (fungal

growth was arrested after appresorium formation) of infection structures was observed after 6 hpi (Figure 7a). No other infection structures were observed after appresorium formation.

Adult plant stage experiments with *P. triticina* race THBL: At the adult-plant stage, the similar growth of the pathogen and disease progress was observed as seen with seedling leaves until 6 hpi. Similar to the seedling stage early abortion of infection units were observed after appresorium formation. However, thin and longer germ tubes were observed (Figure 7b) and appresorium with distorted (flattened and shrunken) shape was observed (Figure 7c).

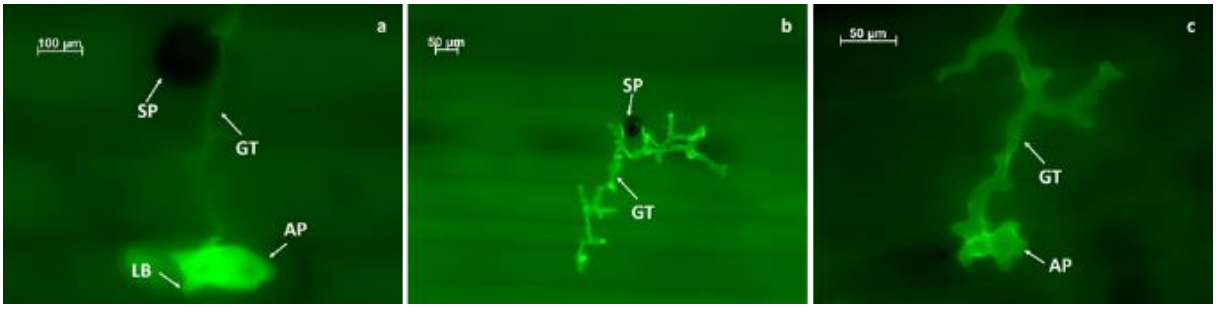

Figure 7. Infection structure development of wheat leaf rust pathogen *P. triticina* on non-host barley genotype Q21861 at seedling and adult-plant stages.

Plants were inoculated with *P. triticina* races THBL and MCDL and examined under an Axio Imager M2 Zeiss Research epifluorescence microscope. Representative images of fluorescence microscopy have shown (**a-c**).

a) Spore (SP) with germ tube (GT) and formation of appresorium (AP) with lobes (LB) by 6 hpi. **b)** An aborted colony with sporelings having two to three thin, branching and long germtube after 6 hpi. **c)** Branching germtube (GT) and appresorium (AP) with distorted (flattened and shrunken) shape in stomata of the plant after 6 hpi.

Seedling plant stage experiments with *P. triticina* races THBL and MCDL: When barley cultivar Harrington was challenged with races THBL and MCDL germ tube (Figure 8a) and appresorium (Figure 8b) formation was observed 6 hpi. Growth and development of the colony similar to wheat cv. Thatcher was observed at up to 96 hpi (Figure 8d). Sporulation was observed by 7 dpi (Figure 8e) with race THBL only. Early abortion of infection structures was observed after appresorium formation when inoculating with race MCDL (Figure 8c).

Adult plant stage experiments with *P. triticina* race THBL: When flag leaves were inoculated with *P. triticina,* germ tube (Figure 8a) and appresorium (Figure 8b) were observed by 6 hpi. Unlike in seedling plant stage, early abortion of infection was observed after 6 hpi and no further growth or development of the pathogen was observed (Figure 8f).

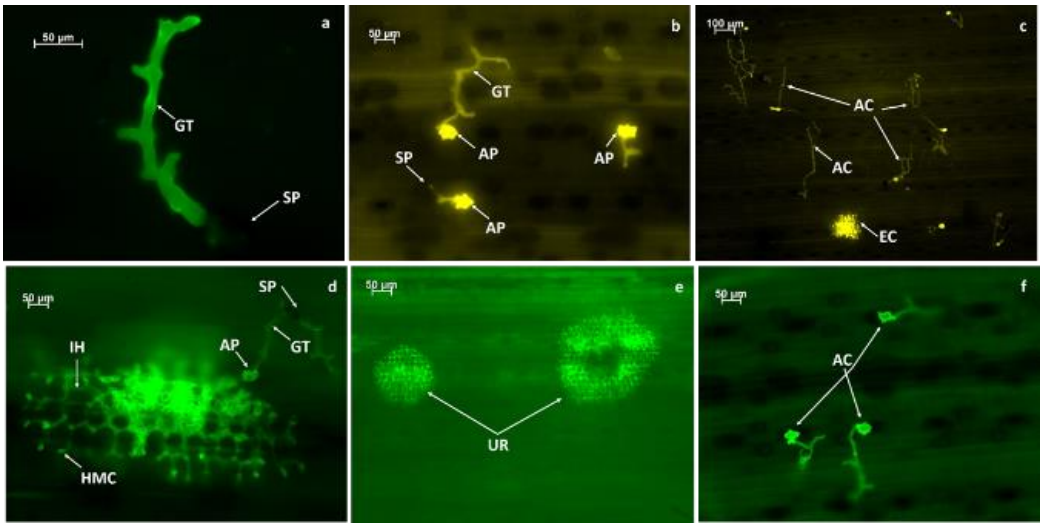

Figure 8. Infection structure development of wheat leaf rust pathogen *P. triticina* on non-host barley line Harrington, at seedling and adult plant stages.

Plants were inoculated with *P. triticina* races THBL and MCDL. The leaf samples were examined under an Axio Imager M2 Zeiss Research epifluorescence microscope. Representative images of fluorescence microscopy have shown (**a-f**).

a) Germinated spore (SP) with branching germ tube (GT) at 2 hpi. **b)** Germ tube (GT) forming appresorium (AP) with lobes (LB) at 6 hpi. **c)** Aborted colony (AC) with sporelings with thin and long germtubes (GT) and Established colony (EC) after 6 hpi. **d)** Infection peg formed from appresorium and sub-stomatal vesicle (SSV) and formation from infection hyphae and Haustorial mother cell (HMC) produced a penetration peg that perforated through mesophyll cell wall and formed a haustorium in the host cell at (24 hpi-96 hpi). **e)** Increase number of haustorial mother cells, uredinium formation (UR) at 7dpi. **f)** Aborted colony (AC) after 6 hpi.

Evaluation of Triticum spp. and Hordeum vulgare accessions for pre-and post-haustorial resistance

A collection of accessions of *Triticum* species and *H. vulgare* were evaluated for pre-haustorial and post-haustorial resistance components by inoculating them with *P. triticina* isolate of race THBL (Table 2). Seedling leaf samples were observed under a fluorescence microscope

to characterize the infection structure development. Susceptible wheat cv. Chinese Spring was used as positive control. Appresorium was observed at 6 hpi in all the genotypes. Fungal growth was arrested after appresorium formation by 6 hpi, and no further growth was observed in *T. monococcum subp. monococcum* accessions PI 119435, PI 428158, PI 518452, PI 95119. *T. monococcum subp. monococcum* accession PI 518452, showed aborted colonies and necrosis at the site of appresorium formation. However, in some of the *H. vulgare* accessions including CIHO 11549, PI 591958, PI 5700, PI 648422, CIHO 11797, CIHO 11765, CIHO 2222 all the infection structures including HMC were observed at 24 hpi and completely established colonies and uredinia with sporulation were observed by 7 dpi (Figure 11 and Table 2).

Table 2. Fluorescence microscopic observations on the seedling leaves of wheat germplasm and barley lines inoculated with urediniospores of *P. triticina* race THBL.

Genotypes	Appresorium	Pre-haustoria	Haustorium	Post-haustoria
Wheat				
PI 119435	1	1	0	0
PI 272560	1	0	1	1
PI 428158	1	1	0	0
PI 518452	1	1	0	0
Barley				
CIHO 11549	1	0	1	1
CIHO 11765	1	0	1	1
CIHO 11797	1	0	1	1
CIHO 2222	1	0	1	1
PI 243182	1	0	1	1
PI 280422	1	0	1	1
PI 483237	1	1	0	0
PI 483237	1	1	0	0
PI 539113	1	0	1	1
PI 5700	1	0	1	1
PI 591958	1	0	1	1
PI 648421	1	0	1	1
PI 95119	1	1	0	0
Chinese Spring	1	0	1	1

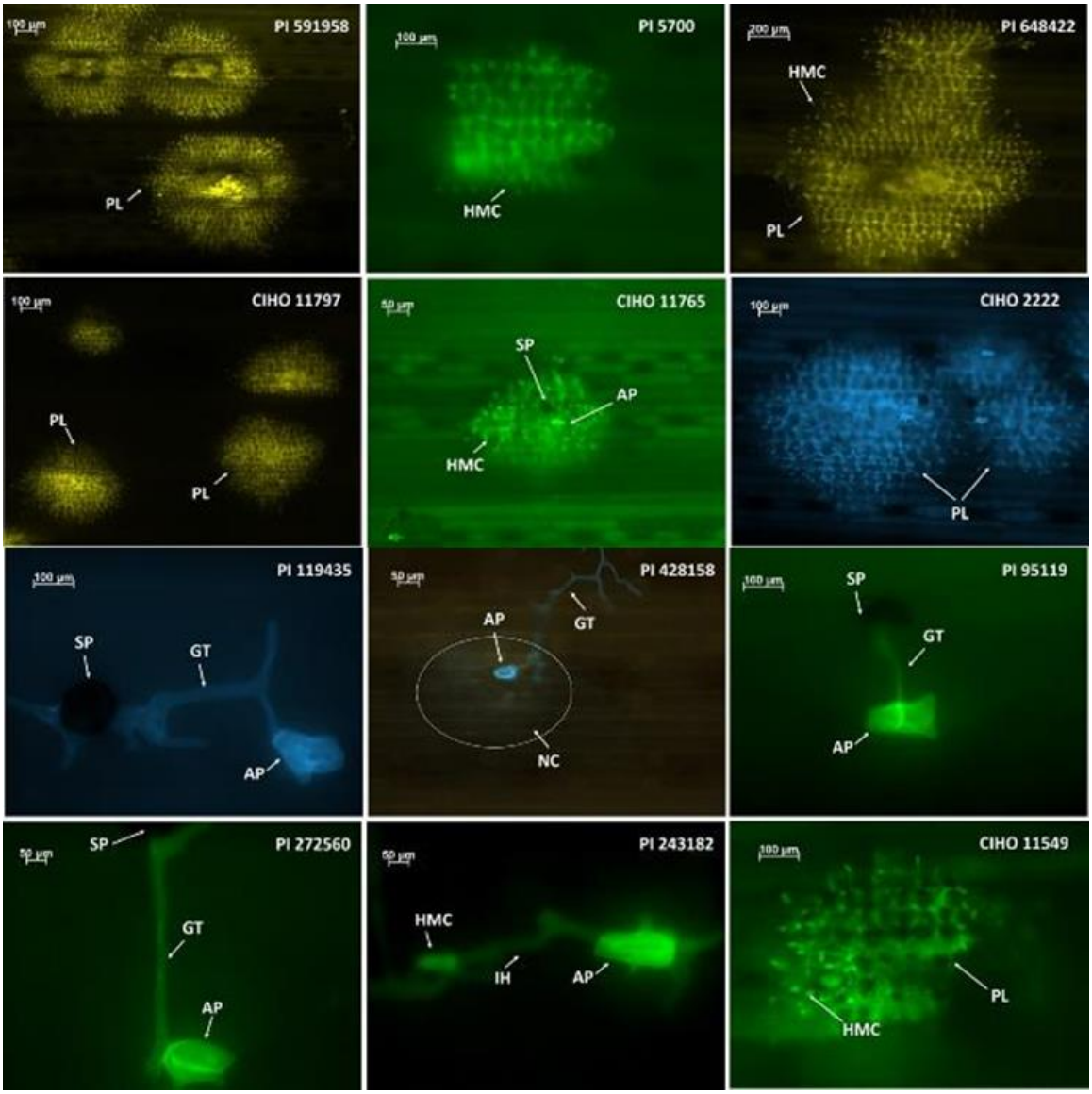

Figure 9. Infection structure development of wheat leaf rust pathogen *P. triticina* in *T. monococcum* subp. *monococcum* accessions and *H. vulgare* spp.

Seedling plant samples of *T. monococcum* spp. and *H. vulgare* spp. accessions PI 119435, PI 428158, PI 518452, and PI 95119 were examined under an Axio Imager M2 Zeiss Research epifluorescence microscope showing Spore (SP) with germ tube (GT) and formation of appresorium (AP) with lobes (LB) by 6 hpi.

Macroscopic observations

Disease symptoms were scored at 14 dpi (Table 3) to evaluate the infection type of wheat NIL'S (Thatcher, Tc-*Lr9,* Tc-*Lr21,* Tc-*Lr34* and Tc-*Lr35*) and barley lines (Q21861 and Harrington) when inoculated with races THBL and MCDL. Susceptible cv. Thatcher had susceptible reaction IT 4 (large uredinia without chlorosis or necrosis) when inoculated with each of the two race at both growth stages (Table 3). Tc-*Lr9,* showed resistant reaction, (IT ;) with visible necrosis, and no uredinia were present when inoculated with each race at both growth stages. Tc-*Lr21* when inoculated with race THBL, showed IT 2 at both growth stages, and when inoculated with race MCDL showed a susceptible reaction with IT3 with uredinia and no necrosis. Tc-*Lr34* showed infection type 3 with uredinia, associated with chlorosis, necrosis, or both when inoculated with each race. Tc-*Lr35* showed IT 3 with uredinia associated with chlorosis with both races while infection type 1 with necrosis was observed when inoculated with race THBL at adult plant stage. Q21861 showed immune (IT=0) resistant reaction, with no necrosis at adult plant stage while IT ';' was observed in seedling plant stage with each race. At seedling plant stage cv. Harrington showed IT 2 with race THBL but displayed IT ';' when inoculated with race MCDL.

Table 3. Macroscopic disease symptoms observed 14 dpi on the seedling leaves of wheat NILs and barley lines inoculated with urediniospores of *P. triticina.*

Genotype	Seedling (THBL)	Seedling (MCDL)	Adult (THBL)
Thatcher	4	4	4
Tc-*Lr9*	;	;	;
Tc-*Lr21*	2+	2	2
Tc-*Lr34*	3	3	2+
Tc-*Lr35*	3	2	3
Q21861	;	;	0
Harrington	2	;	;

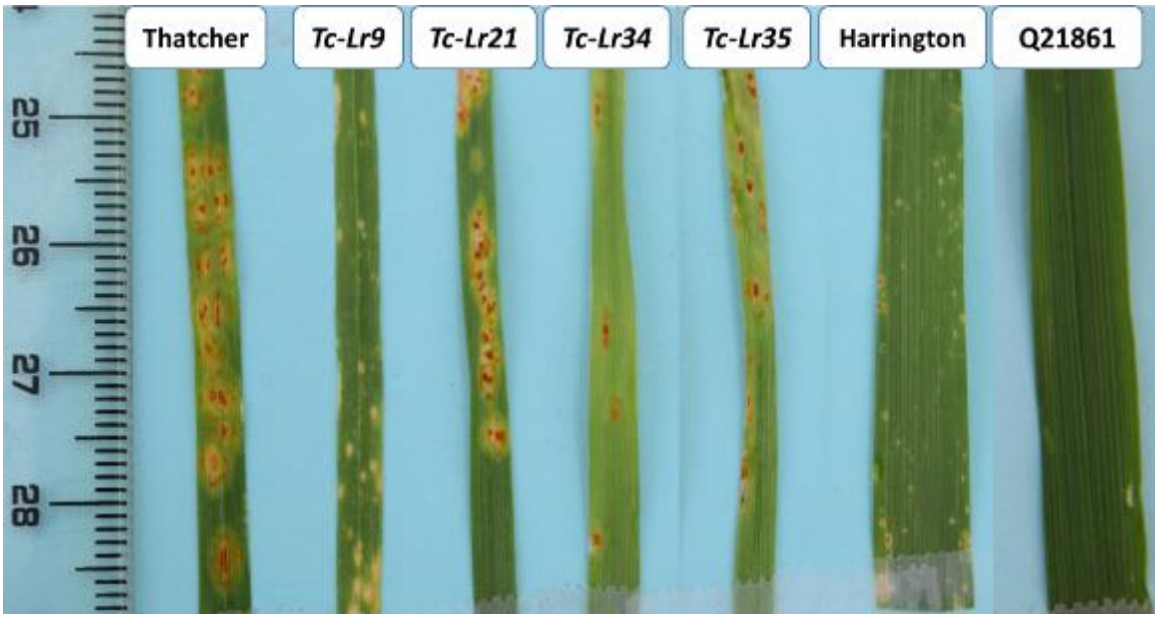

Figure 10. Macroscopic disease symptoms observed 14 dpi on the seedling leaves of wheat NILs and barley lines inoculated with urediniospores of *P. triticina* race **THBL** (Infection type described in Table 2).

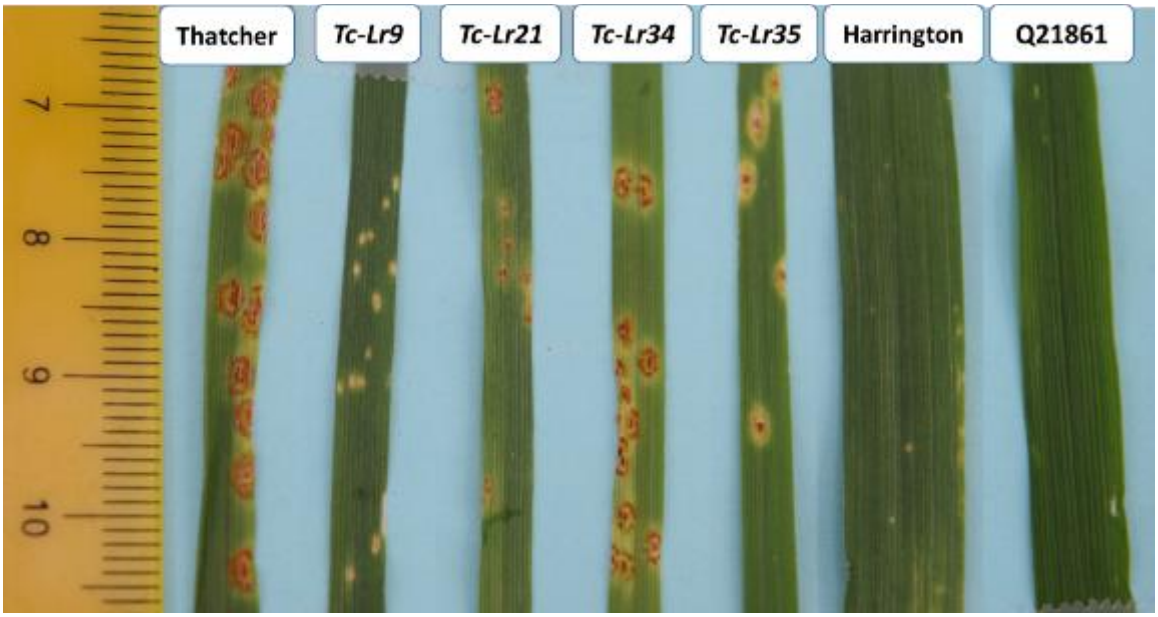

Figure 11. Macroscopic disease symptoms observed 14 dpi on the seedling leaves of wheat NILs and barley lines inoculated with urediniospores of *P. triticina* race **MCDL** (Infection type described in Table 2).

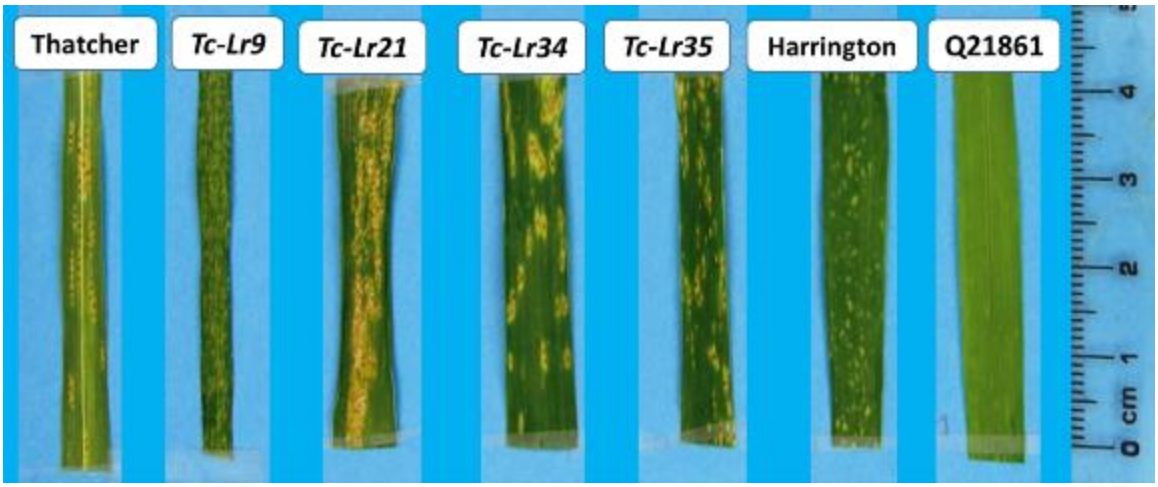

Figure 12. Macroscopic disease symptoms observed 14 dpi on the boot stage leaves of wheat NILs and barley lines inoculated with urediniospores of *P. triticina* race **THBL** (Infection type described in Table 2).

Established colony size (area) measurements in Tc-***NIL's***

Established colonies were observed under a fluorescence microscope at 7 dpi in wheat seedling plant stage samples of susceptible cv. Thatcher and resistant lines Tc-*Lr21*, Tc-*Lr34* and Tc-*Lr35*. Susceptible cv. Thatcher had the largest colony size (area) in comparison to all other NILs when infected with *P. triticina* races THBL and MCDL (Table 4). No area was determined for Tc-*Lr9* as no established colonies were observed in with none of the races (see above section). Tc-*Lr21* colony area was significantly smaller (*p*-value 0.05, Table 4) than susceptible cv. Thatcher and Tc-*Lr34* and Tc-*Lr35*. In Tc-*Lr34*, colony size was significantly smaller than the susceptible cv. Thatcher and larger than Tc-*Lr21* with each race. Colony size of Tc-*Lr35* was not significantly different from susceptible cv. Thatcher with race MCDL. However, Tc-*Lr35* was significantly different from the rest of the NIL's when infected with race THBL. Tc-*Lr35* had larger colony size than Tc-*Lr21* and Tc-*Lr34* (Table 4).

Table 4. *P. triticina colony* size (area) for races MCDL and THBL on wheat cv. Thatcher and its NILs Tc-*Lr9,* Tc-*Lr21,* Tc-*Lr34* and Tc-*Lr35.*

Cultivar	Colony area (sq. microns)	
	MCDL	**THBL**
Thatcher	592583a*	698587 a
Tc-*Lr21*	393400 c	325653 c
Tc-*Lr34*	481061 b	429922 b
Tc-*Lr35*	561089 a	488976 b

* Means with the same letter are not significantly different at p=0.05 using Fishers LSD test. Colonies were measured at 7 dpi using images taken from fluorescence microscopy at 5X magnification.

Discussion

Use of gene pyramiding or gene stacking has been considered as an effective method to achieve more durable and broad resistance. However, it is possible that multiple genes share similar resistance mechanisms and genetic pathways. This study was designed in an attempt to gain insights into the mechanisms involved in leaf rust resistance conferred by various *Lr* genes. Microscopic and macroscopic observations of NIL's and near-non-host genotypes were used to examine resistance components. Spatial and temporal events of infection process and plant responses to *P. triticina* in wheat (Tc-NIL's) and barley (Q21861 and Harrington) at seedling and adult plant stages were characterized with races THBL and MCDL. Based on histopathological study, Tc-NIL's and barley lines could be differentiated into three groups: susceptible (Thatcher), moderately resistant (Tc-*Lr21,* Tc-*Lr34* Tc-*Lr35* and Harrington) and highly resistant (Tc-*Lr9* and Q21861) as early as by 24 hpi. Differences in infection progress, and expression of host defense mechanisms at adult and seedling plant stages in both host and non-host interactions (Thatcher NIL's and among the barley lines) were observed. Leaf rust infection process can be mainly divided into three phases: pre-penetration, penetration and post-penetration. Fungal growth and development can be arrested at any phase of the infection

process and would depend on triggering of various defense resistance mechanisms in host and non-host interactions. In this study, from histological observations, pre-penetration phase seems to be similar in both seedling and adult stages of wheat and barley lines (approximately until 6 hpi) when infected with THBL and MCDL race. In penetration and post-penetration phases (6 hpi-24 hpi), differences in fungal growth and development between seedling and adult stages, between host and non-host interactions as well as between *P. triticina* races THBL and MCDL was observed. This suggests various resistance mechanisms and cellular changes were involved in infection process and also varies in timing of infection. At adult-plant stage of wheat- *P. triticina* interactions and non-host Barley- *P. triticina* interactions, various resistance mechanisms were manifested as abortion of colonies after appresorium formation, and delay in infection structure formation (2 h delay in appresorium and 4 h delay in HMC and haustoria formation) in NIL's (Tc-*Lr21*, Tc-*Lr34*, Tc-*Lr9*) and barley lines (Q21861 and Harrington). In addition, limited growth of the fungus with reduced number of established colonies was observed in adult plant stage of wheat- *P. triticina* interactions in comparison to seedling plant stage. On the *Lr* genes carrying adult plant resistance (APR) Tc-*Lr34* and Tc-*Lr35, P. triticina* developed more rapidly (48 hpi to 96 hpi) at the seedlings than at the adult-stage. Higher number of early abortions occurred in adult-stage in Tc-*Lr21,* Tc-*Lr34* and Harrington (after appresorium formation at approximately 6 hpi) than on seedling when inoculated with the same race. Fungal growth and development was arrested in Tc-*Lr9* and barley line Q21861 with races THBL and MCDL and at both growth stages and is associated with HR (clearly visible as bright cells under fluorescence microscope) after appresorium formation and corresponding to IT (;) at 14 dpi. This could be due to the inability of the fungus to penetrate wheat mesophyll cells. Penetration barriers might include structural differences in primary and boot leaves and morphological

differences in leaves of host and non-host and also might be associated with stomatal closure. Previous research showed that specific chemicals and leaf surface topography may initially determine whether a pathogen can infect a genotype or not (Allen et al. 1990, Collins et al. 2007, Grambow, 1977). Even if the fungus was able to penetrate wheat mesophyll cells, attempted penetration might induce the thickening of cell wall and papillae formation (Ayliffe et al. 2011) as previously studied in rice- *Puccinia graminis* f. sp *tritici, P. triticina, P. striiformis*, and *P. hordei* interactions where, rust can colonize the rice stomatal cells and even able to penetrate the non-host (rice mesophyll cells). Non-host resistance responses were activated and that involves callose deposition, production of reactive oxygen species, and, occasionally, cell death might have arrested the growth and development of penetration pegs. Haustorium induced rapid cell death might also inhibit fungal growth and reduced number of colonies as observed in the previous study of non-host interactions of wheat- *Uromyces fabae* (Jacobs et al. 1996, Hongchang et al. 2012). In the current study, barley genotype Harrington-permitted haustoria formation of *P. triticina* race THBL. This is of interest as *P. triticina* is not considered a barley pathogen. However, previous reports have shown that pathogens infecting non-host can successfully colonize and produce appresorium and even can penetrate the non-host and was able to form SSV, HMC one or two haustorium but, no sporulation (Li et al. 2012, Ayliffe et al. 2011, Zhang et al. 2011, Cheng et al. 2012). Moreover, it is interesting that the level of compatibility is race specific as there was no established colony and sporulation observed when inoculated with *P. triticina* race MCDL. From these observations, it is clear that non-host interactions share similarities with host interactions in terms of infection structure development (Harrington and Tc-*Lr21*) at seedling stage, and in terms of penetration followed by early abortion as observed in both host, (Tc-*Lr9*) and non-host (Q21861) interactions. However, one limitation of this

comparison is the fact that Tc-*Lr9* is a gene introgressed to *T. aestivum* from *Aegilops umbellulatum,* which is a diploid wild wheat species. In diploid wild wheat species, pre-haustorial resistance has been described previously where rust urediniospores develop one or two HMC and further development of haustoria is arrested (Niks, 1982). In a previous study (Wang et al. 2013) microscopic evaluation of Tc-*Lr9* showed pre-haustorial resistance and induction of an HR and cellular lignification. These results suggest that pyramiding of defense responses mediated by non-host and race-specific resistance genotypes plays an important role in the early recognition and suppression of the pathogen. From this study, on the basis of infection process associated with HR, race-specific resistance mechanism could be breakdown into three types: pre-haustorial race-specific resistance; post-haustorial race-specific resistance; non-host race-specific resistance. In pathogen infection process, if appresorium observed was associated with HR, causing cell death and retarding further fungal growth and development is considered as race-specific pre-haustorial resistance. If pathogen was able to form haustorium and is associated with HR after formation of at least one established colony, is considered as post-haustorial race-specific resistance (in this case we still see uredinium associated with both necrosis and chlorosis). In case of non-host resistance, if pathogen was able to form 1 or 2 haustoria in the non-host and is associated with HR, and immediate retarding of fungal growth and development (no sporulation) is considered as non-host race-specific resistance. If pathogen was able to form appresorium and after that abortion of further fungal growth and development and is associated with no HR (necrosis) is considered as immune. Pre-haustorial race-specific resistance will be triggered where, retardation of pathogen infection structures occurs after appresorium formation and is associated with necrosis. If the pathogen overcomes pre-haustorial race-specific resistance (like pathogen associated molecular patterns (PAMPs) then second type, race-specific resistance

post- haustorial resistance would be triggered where pathogen was able to form haustorium in some infection units and early abortion occurs in of rest of infection units and is associated with necrosis. At this point the third type depends on the pathogen establishment. In this case, fungal growth and development would be arrested in very few sporelings yet there is colonization and establishment of the pathogen occurs and is associated with chlorosis (Table 5).

Table 5. Different types of host defense resistance mechanisms associated with race-specific resistance, on the seedling leaves of wheat NILs and barley lines inoculated with urediniospores of *P. triticina.*

Cultivar	Race-specific resistance
Tc-*Lr9*	Pre-haustorial resistance + HR
Tc-*Lr21*	Post-haustorial resistance + HR
Harrington	Post haustorial resistance - HR
Q21861	Pre-haustorial resistance - HR

In the host vs. non-host interactions, there are differences as well as similarities observed in the infection process. Similarities include early abortion associated with HR and reduced number of colonies and sporulation. Differences include distorted infection structures and delay in stomatal recognition and penetration. Non-host mechanisms can be differentiated from host responses in terms of dis-rupture of infection structures (appresorium) and prevent it from gaining the entry into the host and obtain nutrients. Stacking of resistance mechanisms such as pre-and post-haustorial race specific resistance may provide a valuable source of resistance to effectively control the virulent emerging races. Pathogen recognition and induction of HR by host (prevents pathogen entry) would be as early as approximately 6 hours post pathogen attack, and later if some of the infection units gain entry by overcoming the pre-haustorial race-specific

resistance, pathogen growth and development would be limited by less sporulation and reduced number of colonies (post-haustorial race specific resistance). Stacking of several types of race-specific resistance mechanisms seems to be more durable. Based on these observations, we could conclude that our study revealed the cytological and molecular bases of pre- and post-haustorial resistance in wheat and barley against the wheat leaf rust fungus *P. triticina* as early as 24 hpi, and highlighted the significance of race-specific resistance which can be both pre- and post-haustorial resistance and both are involving HR.

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CHAPTER THREE: REAL-TIME PCR FOR DETECTION AND QUANTIFICATION OF FUNGAL BIOMASS IN WHEAT AND BARLEY INFECTED BY *PUCCINIA TRITICINA*

Abstract

Leaf rust, caused by *Puccinia triticina* (*Pt*) is an important disease of wheat (*Triticum aestivum* L.) causing yield losses. Genetic resistance is the most sustainable method for controlling rust diseases. Now-a-days, evaluation of host resistance and fungal infection is mainly based on a disease infection type (IT) or measurement of rust uredinium number and size. However, these methods provide visual estimation rather than exact measurement of fungal growth. Measuring the fungal growth before exhibition of symptoms seems to be one of the preferred methods to evaluate disease resistance. This report describes an in planta quantitative assay for fungal biomass based upon the ratio of Cq values of Host DNA (WCq)/Fungal DNA (FCq). Using this assay the growth of *P. triticina* in Tc-NIL's carrying race-specific and race-nonspecific resistance genes and barley lines was assayed. The assay can discriminate between the susceptible and resistance genotypes with *P. triticina* race THBL. In addition, genotypes carrying pre- and post-haustorial resistance were readily distinguished at early phases of infection. The quantification of *P. triticina* growth on Barley *(Hordeum vulgare)* is also demonstrated, with resistant and susceptible cultivars readily distinguished. However, the real-time PCR approach was much faster and convenient in many cases. Therefore, qPCR allows to assess fungal development at early stages of infection and provides a fast result. This assay can be an excellent tool for in planta quantification of *P. triticina* and can be used for assessment of fungal pathogenicity and host resistance.

Introduction

Leaf rust, caused by *Puccinia triticina* (Pt), is one of the most important disease of wheat (*Triticum aestivum L*.) causing significant yield loss due to reduced seed quality and reduction in kernel weight (Kolmer et al. 2009). Use of resistant varieties is the preferred method to control wheat losses to leaf rust (Bolton et al. 2008). There are more than 73 different *Lr* genes available till date however, most varieties have only one or more specific leaf rust resistance genes (Kolmer et al. 2013, McIntosh, 2013 and Park et al. 2014). Leaf rust resistance genes with major effects (*Lr* genes) have been identified and deployed (Park et al. 2014). Many *Lr* genes to leaf rust display race-specific and or partial resistance rather than immunity (Brouwer et al. 2003 and Oliver et al. 2008). Disease assessment in resistance plants to fungal pathogens is important for the development of resistant genotypes (Ayliffe et al. 2014). Plant phenotyping is scored traditionally, and frequently using qualitative estimates of pathogen development or plant disease symptoms, diseased leaf area (DLA), measurement of rust uredinium number and size and infection type scoring (14 days after infection) have been used to estimate rust disease (Acevedo et al. and Simons, 1985). Scoring for the disease resistance before the exhibition of symptoms seems to be one of the preferred methods to evaluate disease resistance (Brouwer et al. 2003). Although these techniques are relatively quick and easy to perform, there are disadvantages associated with these techniques like, these methods only provide to measure the disease symptoms and not the accurate measurement of fungal growth and pathogen colonization (Qi et al. 2002). To overcome these limitations, qPCR assay was developed that is able to actually assess pathogen presence. Detection and quantification of fungal biomass *in planta* plays a vital role in screening for disease resistance especially in the fungal biotrophic pathogen that cause

necrosis without killing the host, the degree of necrosis over time can be used as a measure of resistance.

The objective of this study was to assess fungal development in the early phases of the infection, before symptoms are visible. A pathogenicity assay was conducted based on the relative quantification of *in planta* fungal DNA in infected wheat and barley leaves by real-time quantitative PCR which is vital to significantly differentiate between lines showing different levels of resistance.

Materials and Methods

Plant material and pathogen inoculum

P. triticina races MCDL and THBL was used to evaluate leaf rust using relative q-PCR assay in wheat and barley. Susceptible wheat cv. Thatcher and its near isogenic lines (NIL'S) Tc*Lr9,* Tc-*Lr21,* Tc-*Lr34,* Tc-*Lr35,* and barley lines Q21861 and Harrington were used for the qPCR study. NIL'S developed by P.L Dyck and R.G Anderson of the Agriculture and Agri-Food Canada Cereal Research Centre in Winnipeg (Hayes et al. 1936). These lines were selected for the *in planta* fungal biomass quantification, in race specific (Tc-*Lr9,* Tc-*Lr21,* Tc-*Lr35)* vs. race non-specific resistance genes (Tc-*Lr34*), and host vs. non-host (Tc-*Lr9,* Tc-*Lr21,* Tc-*Lr34,* Tc-*Lr35 vs.* barley genotypes (Harrington and Q21861).

Experimental design

All the experiments were conducted at the North Dakota State University Agricultural Experiment Station Greenhouse Complex (NDAES) in Fargo. The experiment was arranged in a completely randomized design with two replicates and the entire experiment was repeated twice for each race of the pathogen races. For each genotype three seeds planted per cell were considered as an experimental unit replication. Genotypes were planted in a 24-cell trays with

Sunshine® Mix # 1 (Sungro Horticulture Distribution Inc., Quincy, MI, USA) with slow release seedling fertilizer (Osmocote 15-9-12, N-P-K, Everris NA Inc., OH, USA). Susceptible check Thatcher was included in each tray in all experiments.

Inoculations

Seedling plants were grown in trays in the rust-free greenhouse until second leaf stage was reached (primary leaf fully expanded approximately 7-8 -day-old). All experiments were conducted in greenhouses at 20-21^{o}C during day time and 16-18 ^{o}C during the night; daylight was supplemented for a photoperiod of 16h. Single spore isolate of *P. triticina* races THBL, MCDL were used for inoculations (Long & Kolmer, 1989). Inoculum consisted of fresh urediniospores at a concentration of 6.2 x 10^5 spores per ml in Soltrol 170 oil (Phillips Petroleum, Bartlesville, OK, USA). Inoculations were done using a spray inoculator at the primary leaf. Mock inoculations were performed by spraying plants with Soltrol oil without urediniospores. After inoculation plants were incubated in a mist chamber at 100% relative humidity for 16h in darkness and then transferred to a greenhouse compartment. Mock-inoculated leaf samples were placed in a rust-free greenhouse compartment under the same temperature and light regime.

Time course sample collection and disease infection (IT) type rating

Samples were collected for *in planta* fungal biomass quantification. Inoculated leaf samples were collected at 0 min, 6 hpi, 12 hpi, 18 hpi, 24 hpi, 48 hpi, 72 hpi, 96 hpi, and 7dpi for qPCR assay. Disease phenotype based on infection type (IT) was recorded 14dpi following the 0-4 rating scale where infection type 0 is considered immune (no visible sign or symptoms on the plant) and infection type 4 corresponds to large media which are may be surrounded by chlorosis

(Long D.L and Kolmer J.A, 1989). IT 0 to 2 were considered as avirulent (resistant response), while 3 and 4 were considered as virulent (susceptible response).

Quantitative multiplex PCR analysis of P. triticina in-planta growth

Fungal DNA was quantified in wheat seedling plant leaves infected with wheat leaf rust fungus, *P. triticina*. Genomic DNA was extracted using a modified cetyltrimethylammonium bromide method (Zurn et al. 2015 and Stewart et al. 1993). DNA concentration and quality were determined by nanodrop readings at A260 and A280. Quality was evaluated by running an agarose-gel electrophoresis (100V for 45min) of randomly selected samples. Host and pathogen DNA was extracted from wheat cv. Thatcher and *P. triticina* race THBL respectively and used as standards. Five-point serial dilutions of host and pathogen standards were developed using concentrations ranging from 200 to 0.32 ng/µL as described by Zurn et al. (2015) in accordance with MIQE guidelines. A multiplex standard curve was constructed using the ratio of P. triticina-and wheat. Working solution of 30 ng/µL was prepared and a quantitative PCR protocol was used for the quantification of fungal biomass in infected wheat leaves using primer pairs and probes specific to *P. triticina* and wheat. Primers and probes for reference gene for the barley (Hvubi-F1 and Hvubi-R1) and rust primers (ITS1rustF10d and ITS1rustR3c) were developed (Zurn et al. 2015, Table 6). The specificity of these primers was tested against pure wheat and *P. triticina* genomic DNA samples. Multiplex PCR was carried out in a 12.5 µL reaction volume containing GeneAmp PCR buffer (50 mM KCl, 15 mM Tris–HCl pH 8.0; Applied Biosystems), 2.5 mM MgCl2, 187.5 µM dNTPs, 500 nM of each primer, 1 unit of Go Taq DNA polymerase (Promega Corporation, Madison, WI) and 60 ng of DNA. Amplifications were performed in a thermal cycler (Bio-Rad Laboratories C 1000 with an attached CFX96 real-time PCR detection system, Bio-Rad Laboratories Herculus, CA; Applied Biosystems) using the following

temperature settings: initial denaturation step at 94°C for 5 min, then 40 cycles at 94°C for 30 s, 60°C for 30s, and 72°C for 1 min, followed by a final extension step at 72°C for 10 min. The average of the three replicates was used for estimation of the portion of *P. triticina* genomic DNA in infected leaves. Relative fungal DNA was estimated as the ratio of Cq values of Host DNA (WCq)/Fungal DNA (FCq).

Table 6. Barley Ubiquitin and *P. triticina* internal transcriber spacer (ITS) region primers and probes.

Primer/Probe	Sequence
ITS1rustF10d	TGAACCTGCAGAAGGATCATTA
ITS1rustR3c	TGAGAGCCTAGAGATCCATTGTTA
Hvubi-F1	ACTACAACATCCAGAAGGAGTCCAC
Hvubi-R1	GTCGAAGTGGTTGGCGGCCATGAAGGTC
Pt FAM 4c	FAM-TGAAAGAATCATTGTGATTAAGTATACGTGGCATTCT-TAMRA
Hvubi VIC	VIC-CGCCAAGAAGCGCAAGAAGAAGACGTACACC-MGBNFQ

Results

Evaluation of P. triticina growth in infected wheat leaves via qPCR assay

A q-PCR assay was developed to determine if fungal DNA accumulation in plant tissue can be used as a predictor of resistance (pre-haustorial and post-haustorial resistance) and susceptibility. Primers used in this experiment were specific and produced amplifications only from DNA samples corresponding to the primer (i.e. host or pathogen) and cross amplification was not observed between host and fungal primers and probes. Fungal amplification efficiency was similar in both single and multiplex conditions (Figure 15). Log10 transformation of WCq/FCq was conducted to fit the data into normal distribution. Welch's ANOVA was conducted to account for unequal variances between experiments. Post-hoc pairwise treatment comparisons were made using Cochran-Cox t-test.

Leaf samples of each NIL's and barley genotypes infected with race THBL showed no significant difference in the amount of fungal DNA at 6 hpi. At 12 hpi susceptible cv. Thatcher has the highest amount of FDNA and was significantly different from rest of the NIL's while Tc-*Lr9*, Tc-*Lr21* and Tc-*Lr35* which were grouped together and Tc-*Lr34* and barley lines Harrington and Q21861 were grouped together. At 18 hpi susceptible wheat cv. Thatcher is in its own group with the highest amount of FDNA while Tc-*Lr9,* Tc-*Lr21,* Tc-*Lr34* and Tc-*Lr35* were grouped together and barley lines Harrington and Q21861 were significantly different from Thatcher and grouped together (Figure 15). Tc-*Lr9* and barley line Q21861 had the lowest amount of FNDA at 24 hpi.

At 24 hpi genotypes can be separated into three distinct groups based on Log10 WCq/FCq. Between highly resistant genotypes presenting IT types ";" (Tc-*Lr9* and Q21861) group together with the least amount of fungal DNA. Wheat NIL's Tc-*Lr21,* Tc-*Lr34,* Tc-*Lr35* and barley cv. Harrington grouped together with intermediate levels of fungal DNA while susceptible wheat cv. Thatcher was in its own group (Figure 16). At 48 hpi, 72 hpi and 96 hpi susceptible wheat cv. Thatcher can be separated from the resistant genotypes (Tc-*Lr9,* Tc-*Lr21,* Tc-*Lr34,* Tc-*Lr35,* Q21861 and Harrington). At 7 dpi, the highest amount of fungal DNA was observed in susceptible cv. Thatcher, Tc-*Lr21* and Tc-*Lr35* and least amount of fungal DNA was observed in Tc-*Lr9* and barley line Q21861.

When infected with MCDL race no statistically significant differences were observed (based on mean values) in fungal growth and cannot be clearly grouped into resistant or susceptible clusters until 7 dpi. At 7 dpi, there is a clear difference (based on mean values) in fungal growth and genotypes can be grouped into resistant (Tc-*Lr9,* Harrington and Q21861), moderately resistant (Tc-*Lr35*) and susceptible (Thatcher, Tc-*Lr21,* Tc-*Lr34,*) clusters.

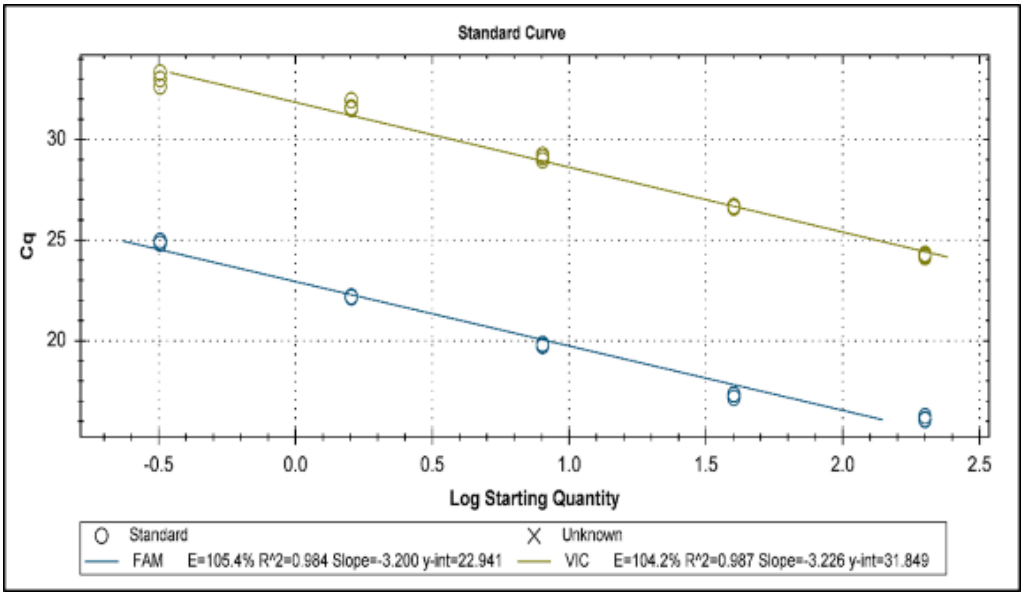

Figure 13. Efficiency curves for the fungal and host DNA standards under single and multiplex conditions.

Fungal: Y =22.94-3.2X, R^2 = 0.984, efficiency = 105.4%; Host: Y =31.849-3.226X, R^2 =0.987, efficiency =104.2%.

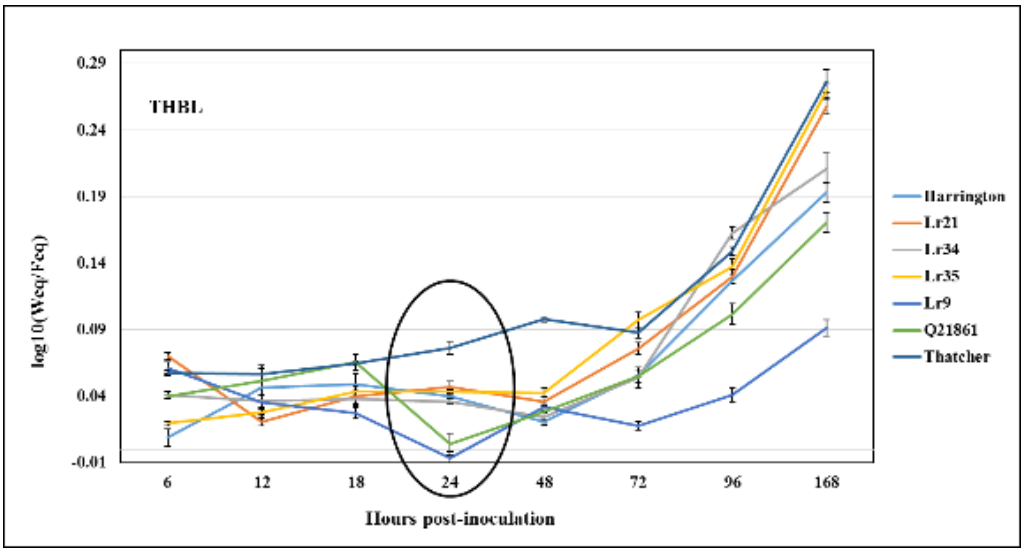

Figure 14. Quantitative real-time polymerase chain reaction (qPCR) assay measuring relative *in planta* FDNA in wheat and barley lines infected with *P. triticina* race THBL.

A time course *P. triticina* growth on leaves of the susceptible *cv.* Thatcher and the resistant genotypes (Tc-*Lr9*, Tc-*Lr21*, Tc-*Lr34* and Tc-*Lr35*) and barley lines (Q21861 and Harrington). Each data point is the average of three technical replicates, each containing 60 ng of homogenate derived from two seedling leaves.

Discussion

During the past several years, the wheat– *P. triticina* interaction has been extensively studied because of its significant impact on yield loses worldwide. Although attempts have been made to detect and quantify the rust fungus using qPCR assay (Wang et al. 2013, Fraaije et al. 2001, Pan JuanJuan et al. 2010 and Zurn et al. 2015), on wheat- *P. triticina* and wheat- *P. striiformis* and wheat- *P. recondita* Barley- *P. recondita* interactions mainly focused on resistance screening, diagnosis, detection and monitoring of the pathogen. No adequate method has been developed to evaluate the time course quantification of *P. triticina* in planta in race-specific resistance genes vs. race-nonspecific resistance genes and host vs. non-host. To our knowledge, this is the first time that both, host and non-host, were utilized and directly compared for the quantification of a fungal plant pathogen.

In this study, we have evaluated the real-time PCR assay for quantification of *P. triticina* in wheat and barley plants to assess fungal development in the early phases of the infection. Our results show that qPCR assay was able to differentiate susceptible and resistant genotypes as early as 24 hpi (before the exhibition of symptoms). According to Wang et al. (2013) in Tc-*Lr9* vegetative growth of *P. triticina* was inhibited completely and no amplification of FDNA was detected in samples collected after 5 dpi. In susceptible lines Thatcher, Tc-*Lr3* and Tc-*LrB* when infected with MBDS race, the highest amount of FDNA was detected and at 5 dpi and increased quickly reached highest by 7 dpi. Similarly, in our study susceptible cv. Thatcher and Tc-*Lr35* have highest amount of FDNA in comparison to the rest of NIL's with race THBL by 7 dpi. Tc-*Lr9* and non-host barley Q21861 has the lowest amount of relative FDNA throughout all the time points and are grouped together showing infection type ";". Therefore, from this study, Tc-*Lr9* is sharing similarities with non-host barley in terms of fungal growth and exhibiting near immune

(very light ;) IT. This is probably due to Tc-*Lr9* gene was introgressed into wheat from *Aegilops umbellulatum* which is a diploid wheat species. Niks, 1982 described a pre-and post-haustorial resistance in diploid species (Niks, 1989) in which pre-haustorial resistance is observed more commonly in non-host interactions (Li et al. 2012, Ayliffe et al. 2011, Hongchang Zhang et al. 2011, and Cheng. Y et al. 2012). Based on the Cq value our qPCR assay could further differentiate the resistant lines Tc-*Lr9* (carrying race-specific resistance gene) and Q21861 into genotypes carrying pre-haustorial resistance as early as at 24 hpi when infected with *P. triticina* race THBL. In addition, Barley (Q21861) - *P. triticina* interactions were investigated through fluorescence microscopy and confirmed the same (Dugyala et al. data un published). Based on Cq value NIL's including Tc-*Lr21* (carrying race-specific resistance) and Tc-*Lr34* (carrying non-race specific resistance) and Barley line Harrington were categorized into a group having post-haustorial resistance. Nevertheless, non-host barley having relative FDNA not significantly different from wheat NIL's with race THBL and exhibition of IT "2" with race THBL and IT ";" with MCDL (showing race-specific resistance) is different from the previous Barley (*Hordeum vulgare*)- *P. triticina* interactions. In the study of Christina et al. (2003) barley lines L94 and Bowman resulted in very susceptible and intermediate susceptible phenotypes that are comparable to wheat control plants when infected with a collection of 56 wheat leaf rust isolates. Similarly, in our study barley line Harrington was moderately susceptible with race THBL and near immune with race MCDL exhibiting race-specific resistance. In addition, Harrington-*P. triticina* interactions were investigated through fluorescence microscopy and confirmed the same (Dugyala et al. data un published). In comparison to the traditional disease rating that is IT, uredinium size and number, the DNA-based real-time PCR assay permits accurate measurement of relative growth and absolute biomass of *P. triticina*. Using qPCR assay, genotypes with

different levels of resistance also can be more accurately evaluated. Due to its high sensitivity and reproducibility, the qPCR method will be ideal for detecting resistance or susceptibility and in addition can be used to differentiate between genotypes carrying various resistance mechanisms (like pre-and post-haustorial resistance). Real-time PCR can be rapidly performed (approximately within 3h) therefore, it is highly useful for processing a large number of samples in a short period of time. In contrast, image analysis through fluorescent microscopy (histo-pathology) to differentiate genotypes carrying pre-and post-haustorial resistance is relatively slow and more labor intensive. However, histo-pathology may have the advantage to study the infection process and various resistance mechanisms involved in host and non-host during disease resistance responses. In this case, disease resistance (e.g., relative growth of *P. triticina*) can be accurately quantified and was coincided with results from image analysis using the epifluorescence microscopy method.

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CHAPTER FOUR: GENE EXPRESSION PROFILING IN *TC-LR21*: UNDERSTANDING THE HISTO-CHEMICAL AND MOLECULAR BASIS OF HYPERSENSITIVE REACTION INVOLVED IN PRE- AND POST- HAUSTORIAL RESISTANCE TO *PUCCINIA TRITICINA*

Abstract

Stacking of resistance genes has been suggested as the preferred practice to achieve broader and longer lasting rust resistance in wheat cultivars. However, little is known about the cellular and molecular mechanisms underlying the resistant phenotypes. Moreover, different genes utilize various pathways that result in resistance to a specific race or multiple races. In the current study, histochemical and gene expression studies were used to determine if HR associated with pre- and post-haustorial resistance was the product of hydrogen peroxide (H_2O_2) accumulation. Susceptible cultivar Thatcher near isogenic lines (NILs) carrying different *Lr* genes were evaluated in a time course experiment. *Puccinia triticina* (*Pt*) inoculated leaf tissue was collected at 0, 1 hpi, 2 hpi, 3 hpi, 4 hpi, 5 hpi, 6 hpi, 12 hpi, 18 hpi, 24 hpi, 48 hpi, 72 hpi, 96 hpi and 7 dpi. Despite carrying pre-haustorial resistance, Tc-*Lr9* presented HR as early as 6 hpi. Tc-*Lr21* presented HR at two different time points, before (5 hpi) and after haustoria formation (24 hpi). HR observed in Tc-*Lr9* indeed involved H_2O_2 accumulation and up-regulation of hypersensitive induced resistance genes (*HIR*) *TaHIR1* and *TaHIR2* at 18 hpi and 24 hpi. The previously cloned and characterized *Lr21* gene, present in Tc-*Lr21* was only up-regulated after haustoria formation. The data obtained from this study provide opportunities to assess components of different resistance mechanisms and suggest that some previous assumptions about plant-pathogen interaction in the host and non-host systems involving pre-haustorial resistance should be revisited.

Introduction

Leaf rust of wheat (*Triticum aestivum* L.) is the most important foliar disease of wheat worldwide. Leaf rust occurs wherever wheat is grown and is most widely distributed of all cereal rusts causing severe yield losses and significantly reducing seed quality (Bolton et al, 2008). Use of resistant varieties is the primary means of rust disease management strategy as it is more economical to the farmers (Martinez et al, 2001). To date, 71 wheat leaf rust resistance (*Lr*) genes have been designated (Kolmer, 2013) but only a few are effective against the highly diverse North American *P. triticina* population and most of these genes show race specific resistance (single plant may be resistant to given rust races and susceptible to others). The race-specific resistance of wheat to *P. triticina* is primarily post-haustorial resistance. However, from recent studies race-specific resistance to leaf rust pathogen to wheat involves two types of resistance; pre-haustorial and post-haustorial resistance (Wang et al. 2012, and Dugyala et al. un published). Rust infection can be arrested at any point in the infection process, from the moment that the spore lands on the plant tissue to after haustoria are formed within the infected host cell (Anker and Niks, 1991). Based on the formation of haustoria by a pathogen in the host cell, previous histological examination of wheat- *P. triticina* interaction has revealed pre- and post-haustorial resistance components (Niks & Dekens, 1991). In pre-haustorial resistance, there is no compatibility exists between the plant and the pathogen and pathogen is not able to form all the infection structures and thus do not complete the disease cycle. The growth and development of the pathogen are arrested after appresorium formation. From recent studies, pre- haustorial resistance does involve Hypersensitive response (HR) in both host and non-host interactions (Wang et al. 2012, Orczyk et al. 2010, Dugyala et al. un published, Ayliffe et al. 2011, Zhang et. al. 2011, Niks, 1983 andCheng, et al. 2014). On the other hand, post-haustorial resistance is a

most common expression of incompatibility of gene-for-gene interactions and is expressed after the formation of at least one haustorium and is associated with HR. Programmed cell death (PCD) or HR is a common defense reaction of host plants to the attack of pathogens. HR is associated with localized, rapid cell death at the site of pathogen attack. Responses triggered by rust fungi during HR involves a wide range of mechanisms, including cell wall lignification, stimulation of lipoxygenase activity, transcription of phenylalanine ammonia-lyase (PAL), pathogenesis related (PR) genes (Beissmann et al. 1992). At the cellular level, changes in the appearance of plant nucleus and cessation of cytoplasmic streaming and changes in the cytoskeleton, cytoplasmic calcium levels, and late generation of oxidative burst occur at plasma membrane which produces reactive oxygen species (ROS) (Wojtaszek, 1997). ROS include hydrogen peroxide (H_2O_2), super oxides ($O2^-$) and hydroxyl ions (*HO). Among all, H_2O_2 plays an important role in host defense mechanism, involves in systemic acquired resistance (SAR) and HR (Vanacker et al. 2000**)**. H_2O_2 can trigger PCD in dose-dependent manner. According to Valerie et al. (2001), low doses of H_2O_2 accumulates in the plant cells in the present of stress conditions while, high doses (50mM of H_2O_2) of H_2O_2 accumulates during pathogen attack. During the HR, several genes have been shown to be activated leading to suppress the pathogen in and around infected cells (Kombrink et al. 1995). Hypersensitive-induced reaction (HIR) proteins family have been identified in cereals including rice (Liang et al. 2010), maize (Nadimpalli et al. 2000), barley (Rostoks et al. 2003), wheat (Yu et al. 2008, Zhang et al. 2009, and Yu et al. 2013) and in other crops including tobacco by functional screening method (Karrer et al. 1998), Arabidopsis (Qi et al. 2011) and pepper (Jung et al. 2007). Eight novel HIR genes have been isolated from maize, including *ZmPHB1, ZmPHB2, ZmPHB3, ZmPHB4, ZmSTM1, ZmHIR1, ZmHIR2 and ZmHIR3* based on amino-acid homology to the tobacco NG1 sequence.

Studies on maize indicated a higher level of expression of *ZmHIR3* gene in Les9 when compared to wild type (Nadimpalli et al. 2000). In barley four HIR genes have been isolated including *HvHIR1, HvHIR2, HvHIR3* and *HvHIR4* based on DNA and amino-acid homologies to maize HIR genes (Rostoks et al. 2003). Amino-acid sequence analysis of barley HIR genes indicated the presence of protein-domain characteristic for prohibitins and stomatins which involved in ion channels and cell cycle (Rostoks et al. 2003). Three *HIR* protein genes in wheat, *TaHIR1, TaHIR2 and TaHIR3*, have been cloned. Studies on wheat *TaHIR1* and *TaHIR3* genes showed inducing HR and regulating defense-related genes. High amounts of transcripts from 24-48 hpi when infected with stripe rust and was reduced by abiotic stress including low temperature, drought, and high salinity. (Yu et al. 2008, Zhang et al. 2009, and Yu et al. 2013). Cloning of the race-specific resistance gene *Lr21*, in *T. aestivum* revealed a NBS- LRR gene (Nucleotide binding site-Luicine rich repeats) regions typical for most disease resistance genes in plants (Huang et al. 2003). Genetically, NBS-LRR class are the most represented group of resistance genes, very numerous in the plant genome, and often occur in clusters at specific loci following gene duplication and amplification events (Daniela et al. 2013). LRRs of plant R proteins are determinants of response specificity, and their action can lead to plant cell death in the form of HR (Huang et al. 2003). To date large number of NBS-encoding sequences have been isolated from various plant species through genome-wide analysis, genome sequencing of Arabidopsis and rice revealed the presence of 149 NBS-LRR-encoding genes in which eleven genes have been identified as functionally active disease resistance genes (Meyers et al. 2003, and Madsen et al. 2003) and 319 genes in soybean genome (Yang et al. 2012).

Stacking of resistance genes has been suggested as the preferred practice to achieve broader and longer lasting rust resistance in wheat cultivars. However, little is known about the

cellular and molecular mechanisms underlying the resistant phenotypes. Moreover, different genes utilize various pathways that result in resistance to a specific race or multiple races. In this study, the accumulation of H_2O_2 during incompatible interactions of Tc-*Lr9* and Tc-*Lr21* with *P. triticina* was investigated using a Histo-chemical protocol and the gene expression profile of known wheat hypersensitive induced resistance genes *TaHIR1* and *TaHIR2* genes.

Materials and Methods

Plant material and pathogen races

To characterize the infection process of wheat leaf rust pathogen *P. triticina*, and to determine the presence of H_2O_2 during incompatible interactions, wheat cv. Thatcher and its NIL's Tc-*Lr9* and Tc-*Lr21* (both carrying race-specific resistance) were used. Tc-NILs were developed by P. L Dyck and R.G Anderson of the Agriculture and Agri-Food Canada Cereal Research Centre in Winnipeg (Hayes et al. 1936). These lines were selected for the evaluation of infection process and to detect the HR in genotypes carrying pre (Tc-*Lr9*)-and post-haustorial resistance (Tc-*Lr21*). Single spore isolates of *P. triticina* races THBL, TNBJ and TDBJ+*Lr21* (Table.7) were used for inoculations (Long & Kolmer et al. 1989).

Table 7. Showing Virulence/avirulence of *P. triticina* races, on wheat cultivars: Thatcher, Tc-*Lr9* and Tc-*Lr21*.

Race	**Thatcher**	Tc-***Lr9***	Tc-***Lr21***
THBL	Virulent	Avirulent	Avirulent
TNBJ	Virulent	Virulent	Avirulent
TDBJ+*Lr21*	Virulent	Avirulent	Virulent

Experimental design

All the experiments were conducted at the North Dakota State University Agricultural Experiment Station Greenhouse Complex (NDAES) in Fargo. The experiment was arranged in a

completely randomized design with three replicates and the entire experiment was repeated twice for each race of the pathogen races. For each genotype three seeds planted per cell were considered as an experimental unit replication. Genotypes were planted in a 24-cell trays with Sunshine® Mx # 1 (Sungro Horticulture Distribution Inc., Quincy, MI, USA) with slow release seedling fertilizer (Osmocote 15-9-12, N-P-K, Everris NA Inc., OH, USA). Susceptible check Thatcher was included in each tray in all experiments.

Inoculations

P. triticina was maintained on the susceptible wheat cv. Thatcher and freshly collected urediniospores were spray inoculated at primary leaf stage wheat seedlings. Urediniospores concentration was adjusted to $6.2x10^5$ spores per ml in Soltrol 170 oil. Inoculated plants were kept in a dark mist chamber (95% RH) for 16h in darkness, and then were returned to the seedling growth conditions at 22–24°C for 16h photoperiod. Mock-inoculation controls were treated the same, except inoculated with Soltrol oil without rust spores. Mock-inoculated leaf samples were placed in a separate, rust-free, greenhouse compartment under the same temperature and light regime.

Time course sample collection and disease infection (IT) type rating

Samples were collected for microscopy, histo-chemical analysis and gene expression study. Non-inoculated leaf samples were collected before inoculation. Mock-inoculated leaf samples were collected at 0 hours post inoculation (hpi). Inoculated leaf samples were collected at 0 min, 1 hpi, 2 hpi, 3 hpi, 4 hpi, 5 hpi, 6 hpi, 12 hpi, 24 hpi, 48 hpi, 72 hpi, 96 hpi, and 7 dpi. Disease phenotype based on infection type (IT) was recorded 14dpi following the 0-4 rating scale where infection type 0 is considered immune (no visible sign or symptoms on plant) and infection type 4 corresponds to large uredia which are may be surrounded by chlorosis (Stakman

et al. 1962, and Long and Kolmer 1989). IT 0 to 2 are considered as avirulent reaction (host resistant response), while 3 and 4 were considered as virulent reaction (host susceptible response).

Sample preparation for microscopic observations and qRT-PCR

Leaf samples were placed in 20 ml disposable scintillation vials filled with leaf clearing solution (chloroform: methanol 3:1 v/v) and placed on ice bath and shaken for 5-6 hours or until the leaves were fully cleared on an orbital shaker (Bio-Express S-3200-Ls Orbital shaker. Variable 115VAC, 60Hz, 0.24amps) for histological study. Leaf samples were collected at 0 min, 1 hpi, 2 hpi, 3 hpi, 4 hpi, 5 hpi, 6 hpi, 12 hpi, 18 hpi and 24 hpi and flash freezed in liquid nitrogen and stored at -80°C until RNA isolation.

Uvitex 2B staining

Mid-leaf tissue segments of about 2cm of three leaves per each entry were harvested and pooled for further histological observations. The leaf segments were processed for fluorescence microscopy using a rapid staining Uvitex 2B protocol (Dugyala et al. 2015). In short, fixing and clearing protocol involved placing each specimen in 60mm glass petri dishes containing 15mL Farmer's fixative (ethanol: acetic acid 3:1 v/v) and shake for 1 hour at 140 rpm on an orbital shaker at room temperature. Specimens were washed with deionized water twice for 10 min each. After staining, specimens were washed once with deionized water for 10 min and stored in 50% glycerol containing a trace (1ml lactophenol in200 ml of glycerol) as a preservative with 50% glycerol and stored at 5˚C in dark conditions until specimens were mounted on microscopic slides. Specimens were examined using an Axio Imager M2 Zeiss Research epifluorescence upright microscope with an excitation filter BP379-401; Chromatic beam splitter FT 420; Emission 435-485; AxioVision rel. 4.8 software. All observations were carried at 20X and 60X.

Three primary leaves each from 3 replicates per cultivar were collected and 6 slides (2 slides per each primary leaf) were mounted for microscopic observations. A total of 10-15 infection units were observed per each slide/per time point/ per cultivar and images of representative infection sites were acquired. Samples with haustoria formed by pathogen during infection process were categorized as carrying post- haustorial resistance and samples with no haustoria formation by pathogen during infection process were considered as carrying pre- haustorial resistance (Niks R.E et al. 1991). Infection sites with all infection structures including germ tube, appresorium, sub-stomatal vesicle (SSV), haustorial mother cell (HMC) and haustorium are considered as established colony and the infection sites with the abortion of infection structures beyond appresorium formation were considered as aborted colonies (Niks, 1982).

Histo-chemical analysis (DAB staining)

Mid-leaf segments of about 2cm of three leaves per each entry were harvested at 0min, 30 min, 1 hpi, 2 hpi, 3 hpi, 4 hpi, 5 hpi, 6 hpi, 24 hpi, 48 hpi, 72 hpi, 96 hpi and 7 dpi. According to a modified procedure of Tada *et al*. 2004 and Wang *et al.* 2007, leaf samples were placed in 1 mg/mL DAB aqueous solution, pH 3.8, and are incubated in the dark for 4 h. Leaf samples were de-stained overnight in ethanol／chloroform (4:1 v／v) containing 0.15% trichloroacetic acid as per a modified version of the procedure in Schweizer 2008. The hydrogen peroxide accumulated in the leaf tissue and converts DAB into a brown insoluble precipitate, which is visible under a light microscope. It allows the detection of hydrogen peroxide production.

RNA isolation and quantitative RT-PCR (qRT-PCR) analysis

Freshly harvested rice leaves were ground with a mortar and pestle that was pre-chilled with liquid nitrogen. RNA samples were isolated from wheat leaves using RNeasy plant mini kit

(Qiagen Inc., Valencia, CA) and further purified with column DNase digestion according to the manufacturer's protocol. Primers for amplification of Tc-*Lr21* (Table 8) were designed.

Table 8. Primers used in qRT-PCR for quantifying the expression of *Lr21* gene.

Primers	**Sequence (5'→3')**
GAPDH-F	TCCTGGGCTT CTCAGTCAAT
GAPDH-R	ATCATACGGCACGCATCAT
TaHIR1-F	CAAGAGGGCTGAAGGTGAGGCAGAATCG
TaHIR1-R	TTTCCAACACTCC TCAAACCATCCTGCC
TaHIR2-F	ATGGGTGGCGTTTTGGGT
TaHIR2-R	TTAGAGAGTATTGGCCTGGAGGA
Tc-*Lr21*-F	GAGTCTATTCCGGTAACTACCAGTG
Tc-*Lr21*-R	GATAGCAGTAAGGACAACATCCAAT

qRT-PCR was performed using iTaq Universal SYBR green supermix (BioRad, Hercules, CA) in BioRad iQTM 5 real-time PCR system following these cycle conditions 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min (Zhang et al. 2009). 25 µl PCR reactions consisted of 12.5 µl 29 SYBR Green PCR Master Mix (Bio-Rad), 10 µM of each primer, 10.5 µl template. Melting curve analyses were performed to detect primer dimerizations. Two independent biological replicates of each sample and three technical replicates of each biological replicate were used for qRT-PCR analyses. The expression level of each gene in different RNA samples was normalized using the GAPDH gene as the internal control (Xiu-Mei et al. 2013). Relative expression levels of each candidate gene were calculated using $2^{-\Delta\Delta Ct}$ method (Liu and Saint 2002). The mean and standard deviation were calculated from three biological replicates.

Results

Infection structures development

The infection process of *P. triticina* races THBL, TNBJ and TDBJ+*Lr*21 in susceptible cv. Thatcher was similar as explained in chapter two in brief, at 4 hpi, appresorium with 3-4 lobes were observed in the stomata on the leaf surface. From the appresorium other infection structures including sub-stomatal vesicle, and infection hyphae and first HMC were observed at 12 hpi (Figure 15a). Number of Haustorial mother cells were observed by 18 hpi with intercellular infection hyphae (Figure 15b). In later time points including 48 hpi, 72 hpi and 96 hpi an increasing number of haustorial mother cells, and colony development was observed (Figure 15c). Finally, by 7 dpi, a completely established colony with matured and newly formed spores were observed in established colonies (Figure 15d).

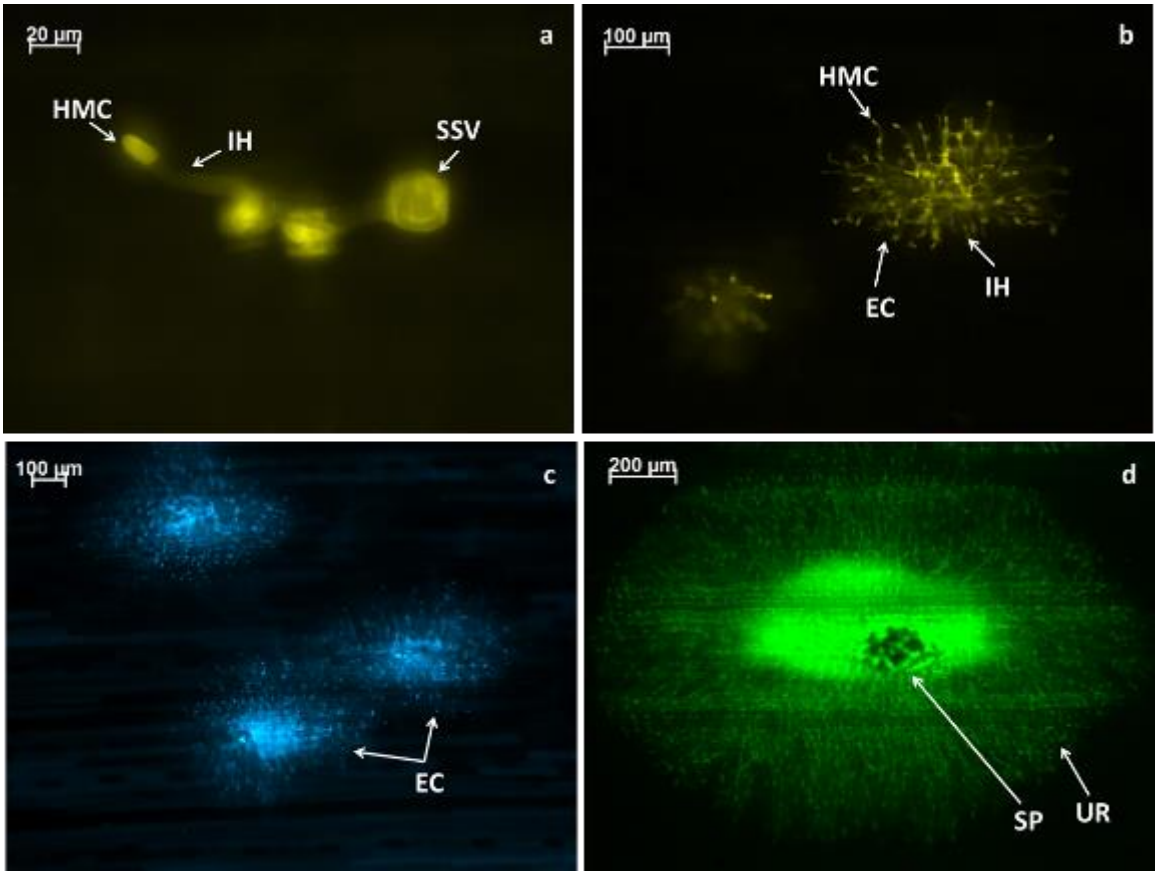

Figure 15. Infection structure development of wheat leaf rust pathogen *P. triticina* on susceptible wheat cv. Thatcher at seedling plant stage.

Plants were inoculated with *P. triticina* races THBL and TNBJ and TDBJ+*Lr*21 and examined under an Axio Imager M2 Zeiss Research epifluorescence microscope (**a-d**). **a)** Sub-stomatal vesicle (SSV) and formation of infection hyphae and subsequent first haustorial mother cell (HMC) by 18 hpi. **b-c)** Increased a number of Haustorial mother cells (HMC) produced and established colony (EC) development by 48 hpi **d**) Uredinium development (UR) with sporulation (SP) by 7dpi.

Tc- *Lr9* + TNBJ: Tc-*Lr9* when inoculated with virulent race (TNBJ), appresorium was not observed prior to 6 hpi and infection structures including infection peg, sub-stomatal vesicle, and infection hyphae were observed at 12 hpi (Figure 16a). First HMC was observed by 18 hpi (Figure 16b). More number of Haustorial mother cells were observed by 18 hpi with intercellular infection hyphae (Figure 16c). Development of established colonies was observed by 24 and 48

hpi (Figure 16d-16e). Completely established colony and sporulation occurred by 7 dpi with virulent (TNBJ) (Figure 16f).

Tc- *Lr9* + THBL: Tc-*Lr9* when infected with avirulent race THBL, appresorium was not observed prior to 6 hpi. Necrosis was observed in the plant cells close to appresorium formation. After appresorium formation, no penetration of the pathogen into the host cells was observed. Fungal growth was arrested (approximately 6 hpi), and there is no colony development or sporulation was observed by 7dpi.

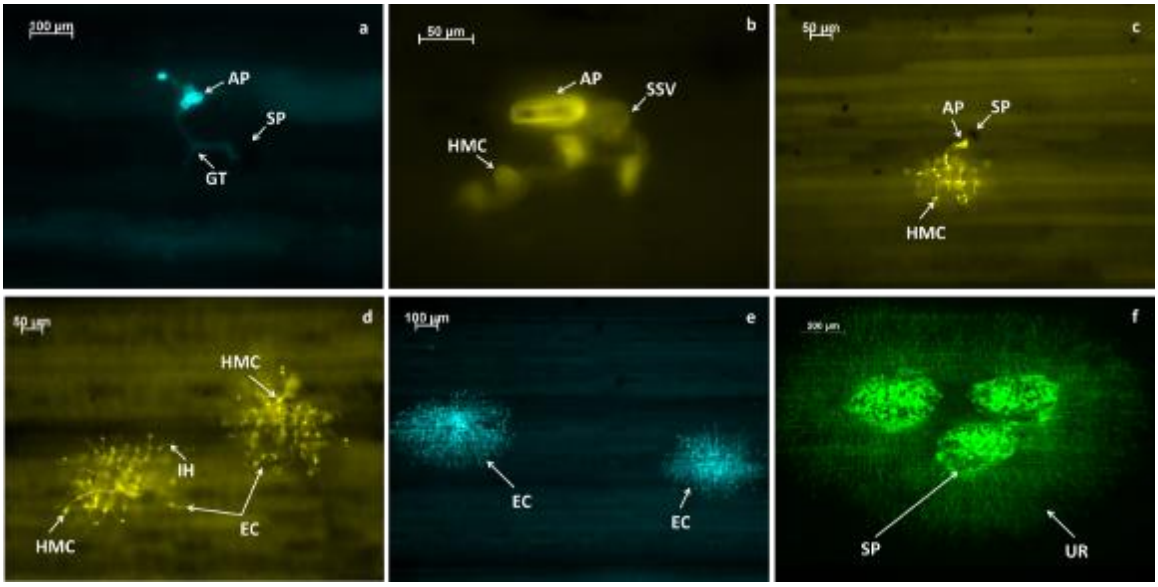

Figure 16. Infection structure development of wheat leaf rust pathogen *P. triticina* on Tc-*Lr9* at seedling plant stage.

Plants were inoculated with *P. triticina* races TNBJ and examined under an Axio Imager M2 Zeiss Research epifluorescence microscope (**a-f**). **a)** Spore (SP) with branching germ tube (GT) and appresorium (AP) by 6 hpi. **b)** Infection peg formed from appresorium and subsequent sub-stomatal vesicle (SSV) and first Haustorial mother cell (HMC) produced from infection hyphae by 12 hpi. **c-d)** Increased in the number of haustorial mother cells and penetration peg that invaginate through mesophyll cell wall to form a haustorium by 18 hpi. **e-f)** Increase number of haustorial mother cells, and uredinium development (UR) with sporulation (SP) by 7dpi.

Tc-*Lr21* + TDBJ: Tc-*Lr21* when inoculated with virulent race TDBJ, urediniospores germination and appresorium formation took place within 2 hpi and 3 hpi respectively, Other

infection structures including infection peg, sub-stomatal vesicle, and infection hyphae were observed at 12 hpi (Figure 17a) and HMC were observed by 18 hpi (Figure 17b). Development of established conies was observed by 24 hpi and 48 hpi (Figure 17d-17e). Completely established colony and sporulation occurred by 7 dpi similar to susceptible cv. Thatcher (Figure 17f).

Tc-*Lr21* + THBL: However, some early abortion of sporelings defined as no further growth and development of fungus after appresorium formation was observed at 48 hpi when challenged with avirulent race (THBL) (Figure 17c).

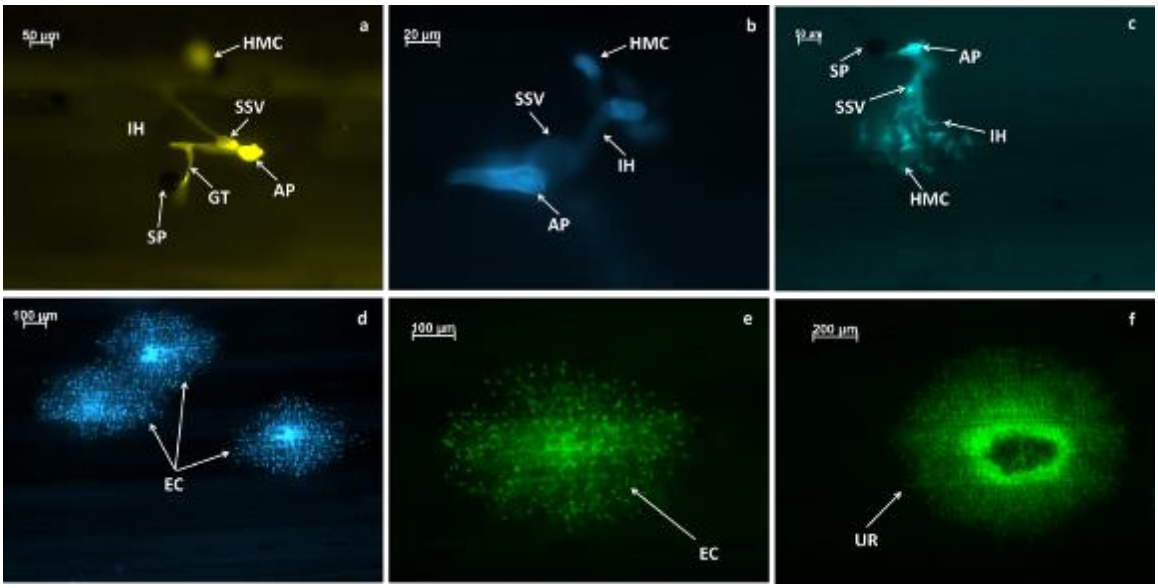

Figure 17. Infection structure development of wheat leaf rust pathogen *P. triticina* on Tc-*Lr21* at seedling plant stage.

Plants were inoculated with *P. triticina* races TDBJ+*Lr21* and examined under an Axio Imager M2 Zeiss Research epifluorescence microscope (**a-f**). **a)** Spore (SP) with branching germ tube (GT) and appresorium (AP) by 6 hpi. **b)** Formation of first Haustorial mother cell (HMC) produced from infection hyphae by 12 hpi. **c)** Increased in the number of haustorial mother cells and penetration peg that invaginates through mesophyll cell wall to form a haustorium by 18 hpi. **d-e)** Increase in a number of haustorial mother cells, and formation of established colony by 24 hpi. **f)** Uredinium development (UR) with sporulation (SP) by 7 dpi.

DAB staining

Wheat *P. triticina* compatible interactions, generally do not involve necrosis/cell death at the site of infection and therefore no H_2O_2 accumulation is observed in and around the fungal infected cells. On the other hand, wheat- *P. triticina* incompatible interaction, avr-R genes generally involve necrosis which results in the accumulation of H_2O_2 in the necrotic (dead) plant cells. H_2O_2 accumulation in the dead plant cells is seen as a brown precipitate when stained with DAB and observed under light microscope. In this study, all the samples were initially stained with DAB and finally, the same samples were stained with Uvitex 2B to confirm the necrosis is due to the presence of infection structures of *P. triticina.*

Thatcher: Susceptible cv. Thatcher inoculated with virulent and avirulent races, was examined under a light microscope to detect the H_2O_2 accumulation. There was no or very low amount of H_2O_2 accumulation was observed at 0 min-24 hpi (Figure 18a-d). Same samples were examined under fluorescent microscope. *P. triticina* infection structures including germ tube, appresorium and haustorium were observed as explained earlier. There was no H_2O_2 accumulation was observed at the later time points (48 hpi, 72 hpi and 96 hpi). At 7 dpi, low amounts of H_2O_2 was observed. Infection structures including established colony and sporulation were associated with low levels of H_2O_2 accumulation at 7 dpi.

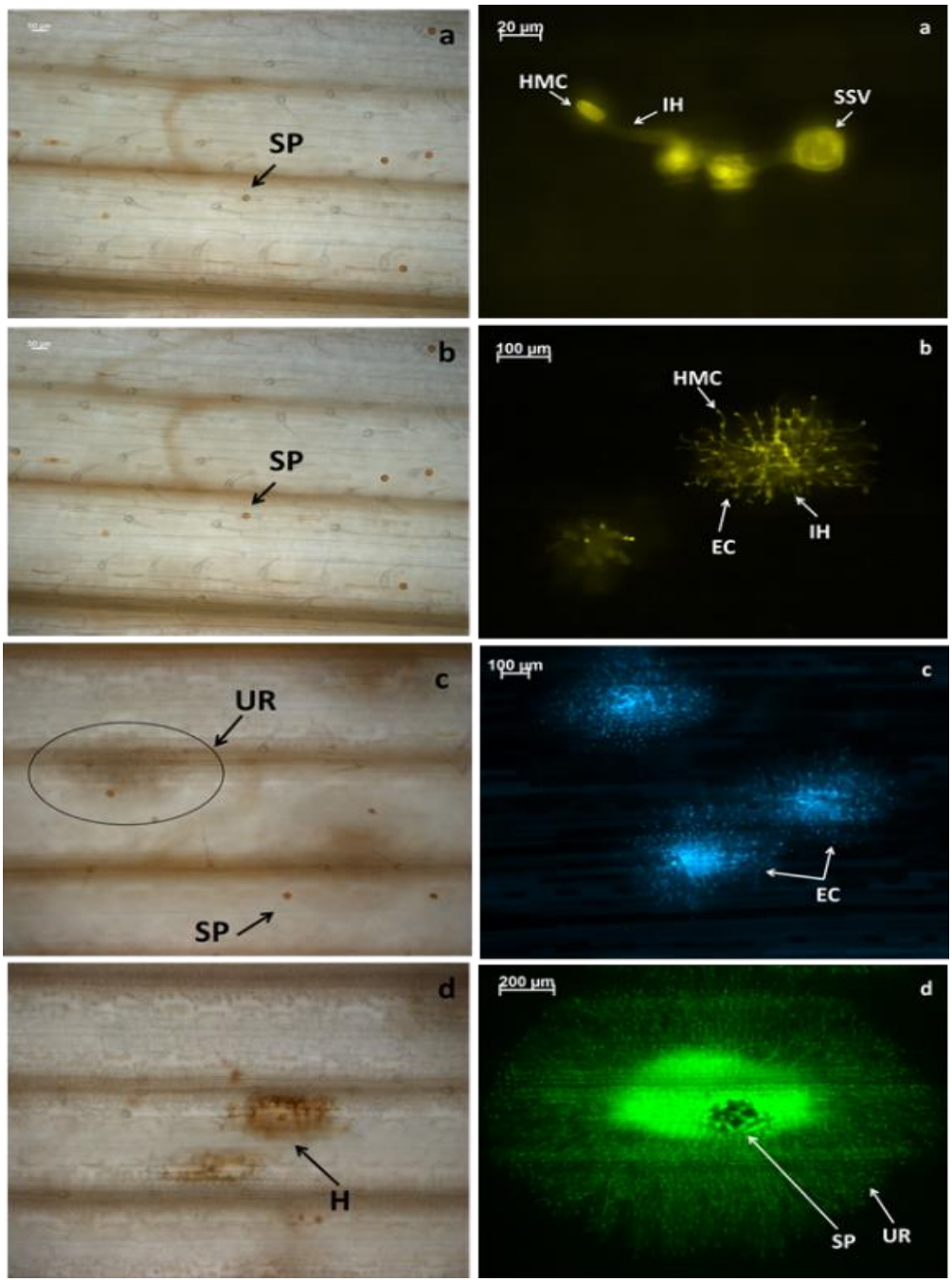

Figure 18. Microscopic localization of hydrogen peroxide (H_2O_2) accumulation in wheat leaves after inoculation with urediniospores of *P. triticina* races THBL at seedling plant stage of wheat susceptible cv. Thatcher.

Images on the left side are leaf samples stained with DAB and observed under a light microscope. Images on the right side are leaf samples stained with Uvitex2B and observed under a fluorescence microscope at the same spot to conform the DAB accumulated is due to the pathogen. Representative images are shown.

Tc-*Lr9*+ TNBJ: H_2O_2 accumulation was observed in Tc-*Lr9* guard cell, epidermal cells and mesophyll cells when inoculated with avirulent race (TNBJ), H_2O_2 accumulation was first observed at 30mpi (Figure 19a) in epidermal cells and cells surrounding fungal infection structures. At 2 hpi, a large amount of H_2O_2 accumulation was observed in epidermal cells and guard cells and surrounding urediniospores and fungal germtube (Figure 19b). At 6 hpi, H_2O_2 accumulation was still observed in epidermal cells and cells surrounding urediniospores, germtube appresorium (in the stomata) (Figure 19c). At 24 hpi, H_2O_2 accumulation was observed in guard cells, epidermal cells and mesophyll cells. (Figure 19d). In the later time points including 48 hpi, H_2O_2 accumulation was observed surrounding appresorium was observed (Figure 19e). At 72 hpi, H_2O_2 accumulation was only observed in pin point spots in epidermal cells surrounding appresorium (Figure 19f). Similarly, at 96 hpi, small amount of H_2O_2 accumulation was observed only in spots and infection structure observed was appresorium (Figure 19g) and 7 dpi low levels of H_2O_2 accumulation was observed when compared with early time points.

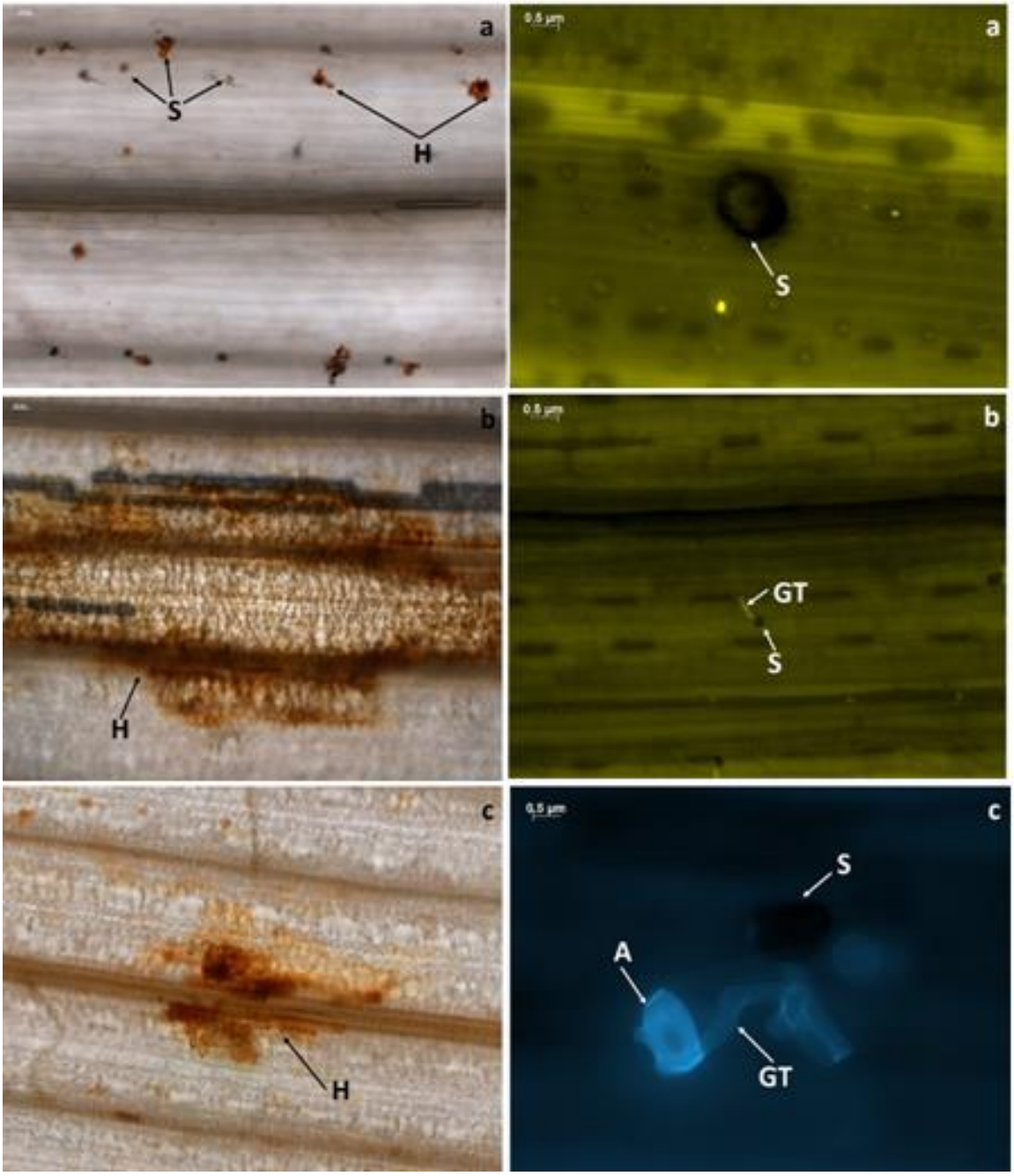

Figure 19. Microscopic localization of hydrogen peroxide (H_2O_2) accumulation in wheat leaves after inoculation with urediniospores of *P. triticina* avirulent race THBL at seedling plant stage of wheat NIL Tc-*Lr9*.

Leaf samples stained with DAB and observed under a light microscope. Images on the right side are leaf samples stained with Uvitex2B and observed under a fluorescence microscope at the same spot to conform the DAB accumulated is due to the pathogen. Representative images are shown.

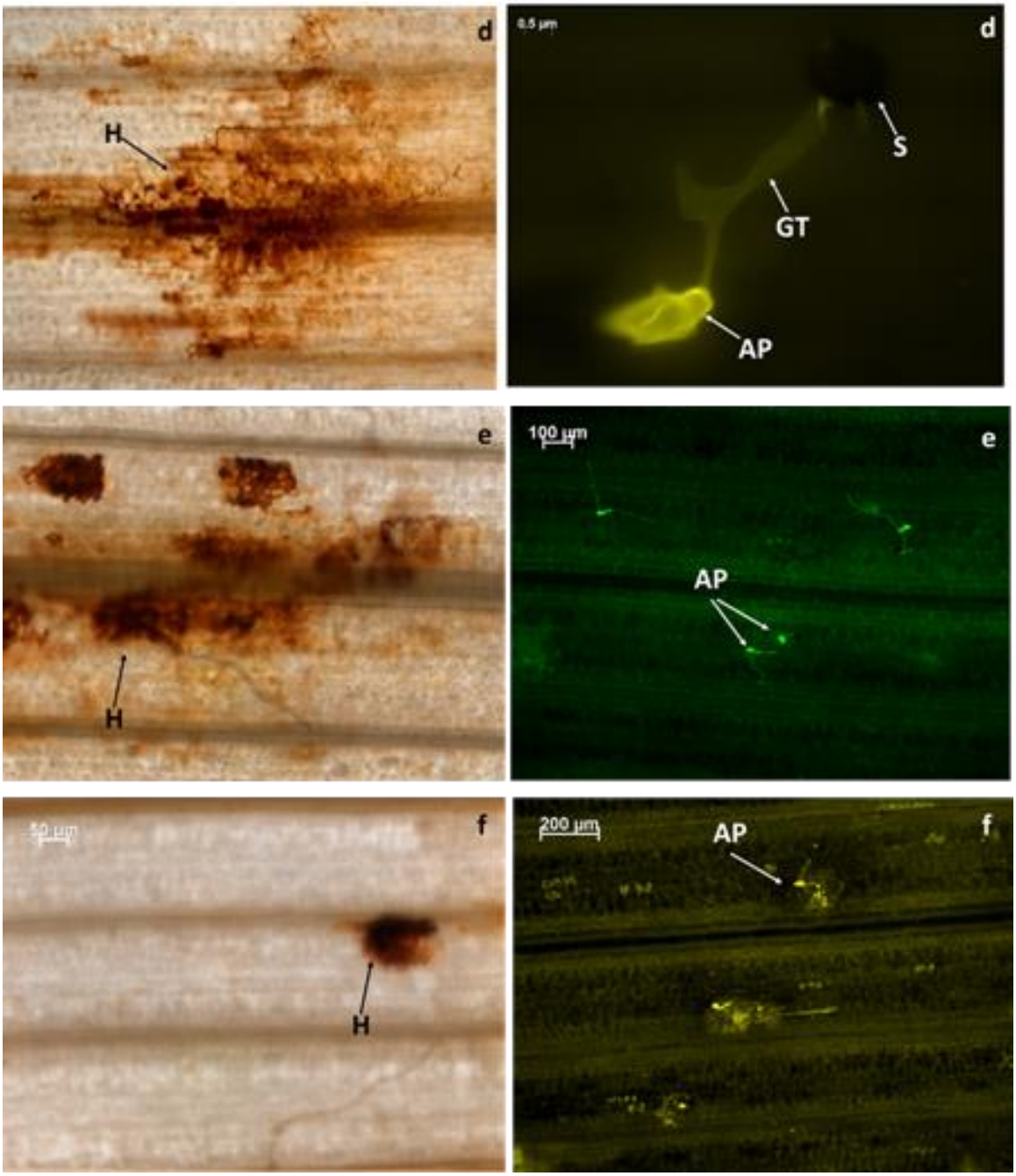

Figure 19. Microscopic localization of hydrogen peroxide (H_2O_2) accumulation in wheat leaves after inoculation with urediniospores of *P. triticina* avirulent race THBL at seedling plant stage of wheat NIL Tc-*Lr9*. (Continued)

Leaf samples stained with DAB and observed under a light microscope. Images on the right side are leaf samples stained with Uvitex2B and observed under a fluorescence microscope at the same spot to conform the DAB accumulated is due to the pathogen. Representative images are shown.

Tc-*Lr9*+TNBJ: Tc-*Lr9* when inoculated with virulent race (TNBJ), there was no or very little amount of H_2O_2 was observed as seen in the susceptible cv. Thatcher (Figure 19).

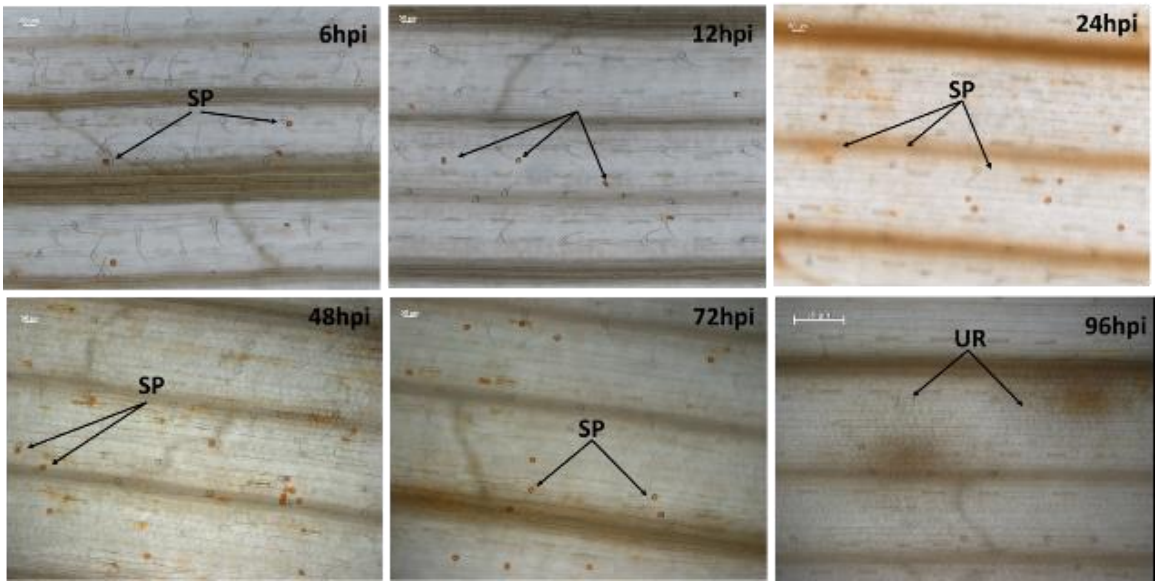

Figure 20. Microscopic localization of hydrogen peroxide (H_2O_2) accumulation in wheat leaves after inoculation with urediniospores of *P. triticina* virulent race TNBJ at seedling plant stage of wheat NIL Tc-*Lr9.*

Leaf samples stained with DAB and observed under a light microscope. Images on the right side are leaf samples stained with Uvitex2B and observed under a fluorescence microscope at the same spot to conform the DAB accumulated is due to the pathogen. Representative images are shown.

Tc-*Lr21+THBL*: In Tc-*Lr21 P. triticina* compatible interaction, H_2O_2 accumulation was observed in guard cell, epidermal cells and mesophyll cells. The first time point at which H_2O_2 accumulation was observed at 3 hpi and then at 5 hpi, 24 hpi and 48 hpi. Infection structures associated with H_2O_2 accumulation were appresorium and haustorium. In the later time points including 72 hpi, 96 hpi and 7 dpi low levels of H_2O_2 accumulation were observed when compared with early time points (Figure 21).

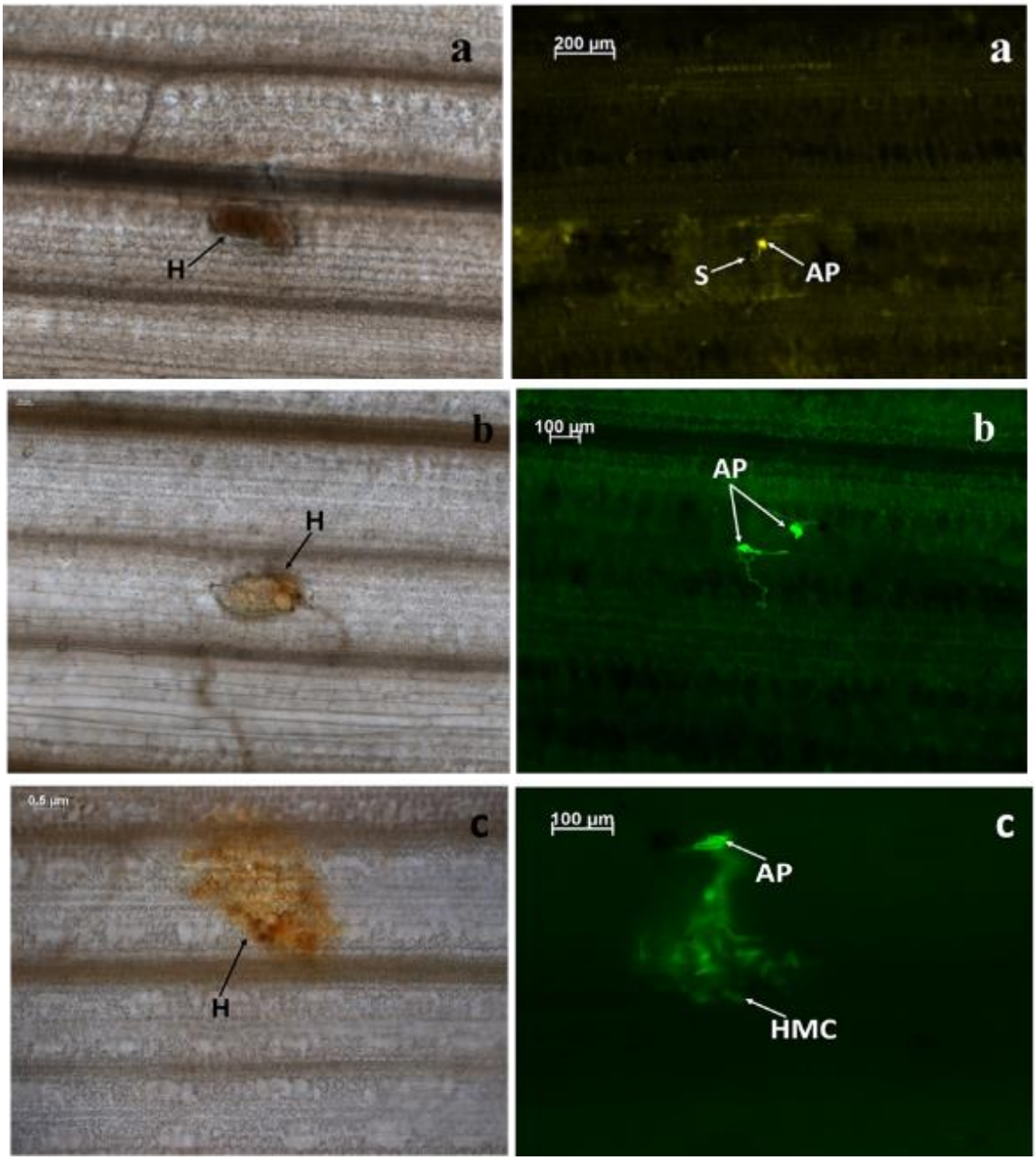

Figure 21. Microscopic localization of hydrogen peroxide (H_2O_2) accumulation in wheat leaves after inoculation with urediniospores of *P. triticina* race THBL at seedling plant stage of wheat NIL Tc-*Lr21*.

Leaf samples stained with DAB and observed under a light microscope. Images on the right side are leaf samples stained with Uvitex2B and observed under a fluorescence microscope at the same spot to conform the DAB accumulated is due to the pathogen. Representative images are shown.

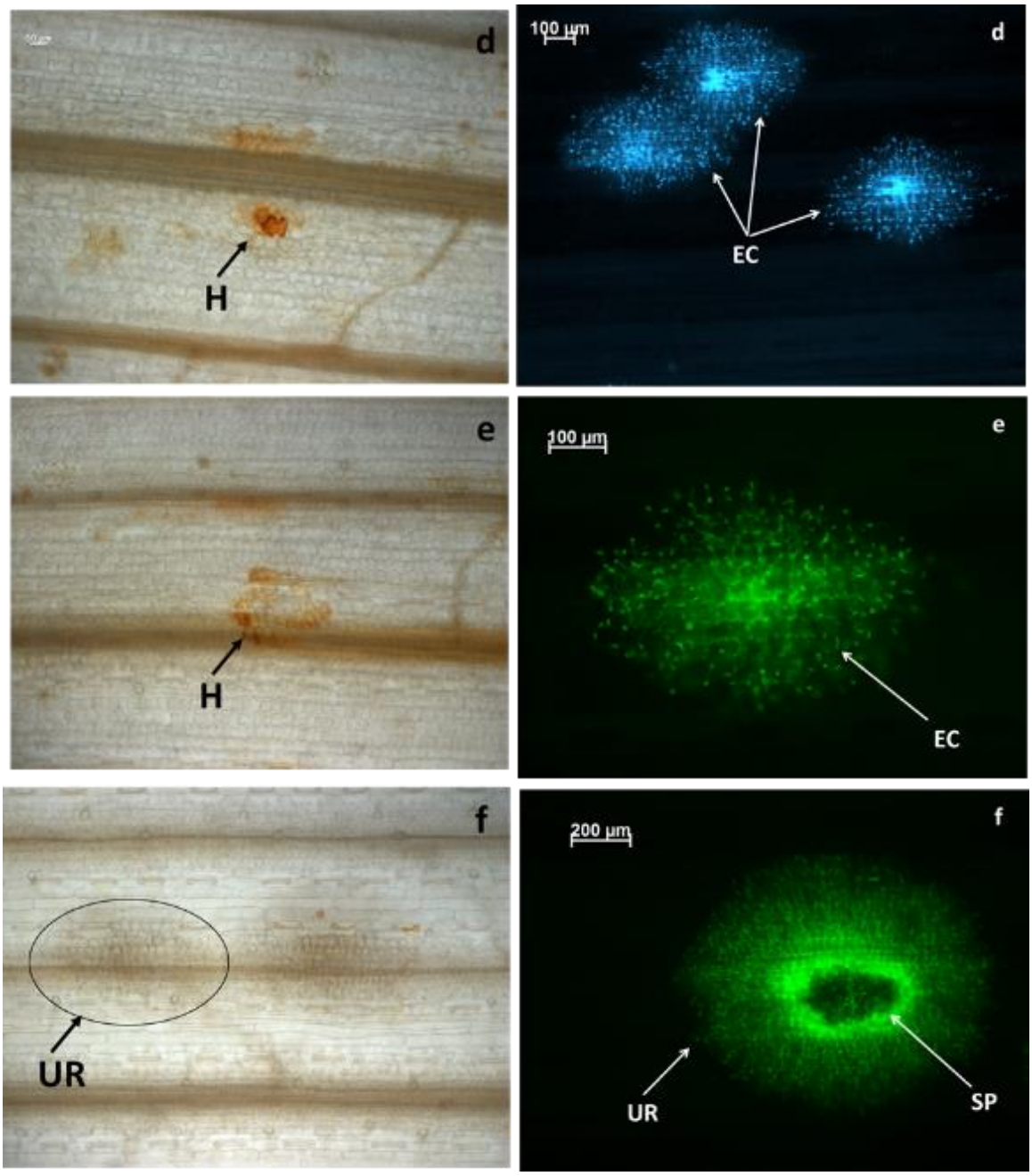

Figure 21. Microscopic localization of hydrogen peroxide (H_2O_2) accumulation in wheat leaves after inoculation with urediniospores of *P. triticina* race THBL at seedling plant stage of wheat NIL Tc-*Lr21*. (Continued).

Leaf samples stained with DAB and observed under a light microscope. Images on the right side are leaf samples stained with Uvitex2B and observed under a fluorescence microscope at the same spot to conform the DAB accumulated is due to the pathogen. Representative images are shown.

Tc-*Lr21+TDBJ*: Cultivar Tc-*Lr21* when inoculated with virulent race, there was no or very low amount of H_2O_2 accumulation was observed as seen in the susceptible cv. Thatcher (Figure 21).

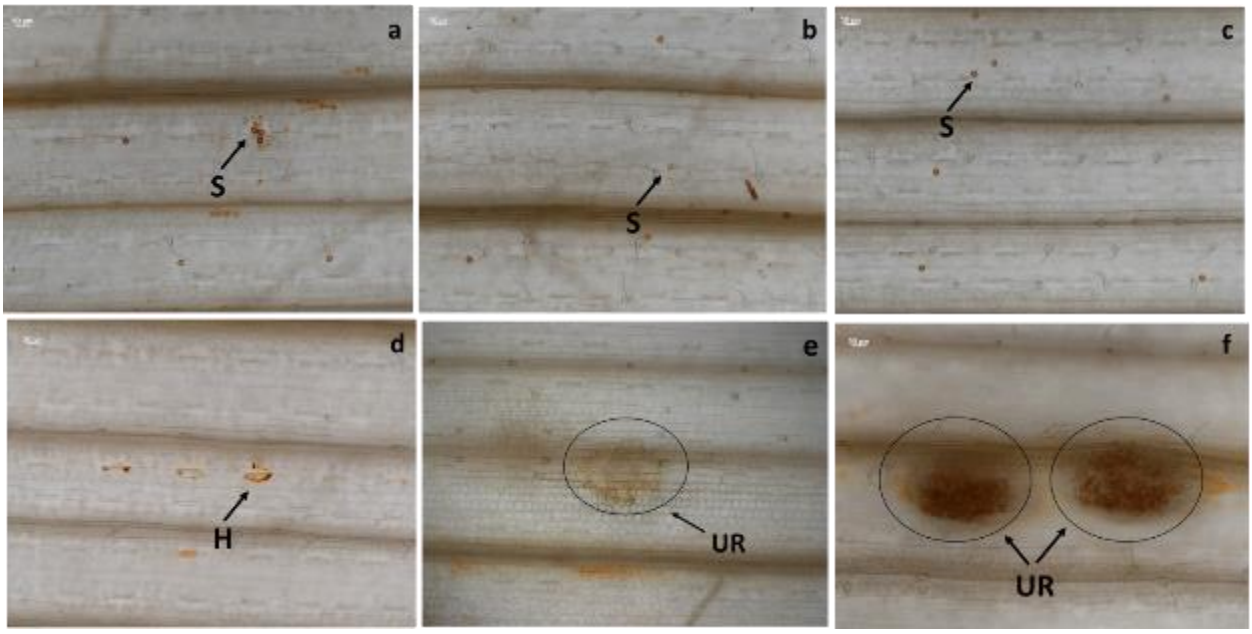

Figure 22. Microscopic localization of hydrogen peroxide (H_2O_2) accumulation in wheat leaves after inoculation with urediniospores of *P. triticina* virulent race TDBJ+*Lr21* at seedling plant stage of wheat susceptible cv. Thatcher.

Leaf samples stained with DAB and observed under a light microscope. Images on the right side are leaf samples stained with Uvitex2B and observed under a fluorescence microscope at the same spot to conform the DAB accumulated is due to the pathogen. Representative images are shown.

Macroscopic observations

Disease symptoms were scored at 14 dpi (Figure 23) allowing to evaluate the infection type on Thatcher, Tc-*Lr9*, and Tc-*Lr21* when inoculated with virulent and avirulent races. Susceptible cv. Thatcher had susceptible reaction IT 4 (large uredinia without chlorosis or necrosis) when inoculated with each race. Tc-*Lr9* showed a susceptible reaction (IT 4) when inoculated with virulent race TNBJ and showed resistant reaction IT ";" when inoculated with

avirulent race THBL. Tc-*Lr21* showed susceptible reaction IT 4 when inoculated with virulent race TDBJ+*Lr21* and showed IT 2+ with no necrosis when inoculated with avirulent race THBL.

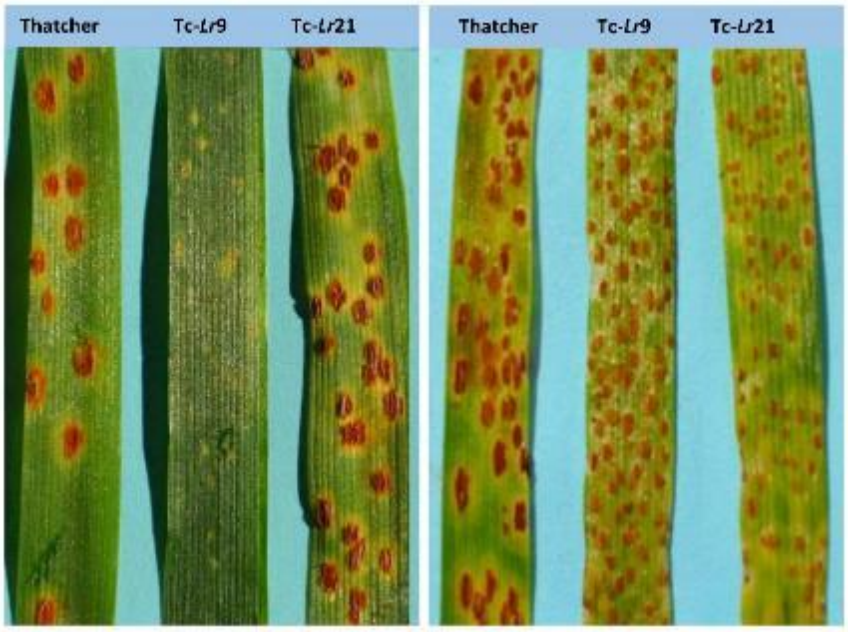

Figure 23. Macroscopic disease symptoms observed 14 dpi on the seedling leaves of wheat NILs inoculated with urediniospores of *P. triticina*.

Assaying for the expression profiles of Tc-***Lr21 gene by qRT-PCR analysis***

To study the expression profile of the transcripts of Tc-*Lr21* gene, transcriptional signatures of susceptible cv. Thatcher and moderately resistant Tc-*Lr21* was compared in response to wheat- *P. triticina* avirulent race THBL infection, in mock-inoculated and *P. triticina* infected leaf material (Figure 24). Mock-inoculated leaf samples were considered as control. The Tc-axis indicates the amount of Tc-*Lr21* gene and was compared to its mock-inoculated samples (Inoculated Thatcher sample was compared to Thatcher mock-inoculated sample and Tc-*Lr21* was compared to Tc-*Lr21* mock-inoculated sample) and the x-axis indicates the different sampling time points. A set of primers based on the cloned sequences of the Tc-*Lr21* gene was designed and qRT-PCR was performed to test their expression profiles during *P. triticina* infection across a series of time points post-inoculation. As shown in Figure 26, there is no

accumulations of Tc-*Lr21* transcripts at 0 hpi, 1 hpi, 2 hpi and 3 hpi as the expression level was below 1-fold when compared to its mock-inoculated samples. The transcripts of Tc-*Lr21* gene was up-regulated as early as 4 hpi when compared to mock-inoculated Tc-*Lr21* sample, and peaked at 5 hpi whose transcripts were 4-fold over that of mock-inoculated Tc-*Lr21* samples. The accumulation of transcripts decreased and declined to original expression levels at 6 hpi, 18 hpi and at 24 hpi (Figure 24). However, a number of transcripts of both Thatcher and Tc-*Lr21* at every time point was higher than that of the mock-inoculated sample. These results indicated that *P. triticina* infection triggered the induction of Tc-*Lr21* gene in Wheat-*P. triticina* interactions peaking at 5 hpi. When susceptible cv. Thatcher and Tc-*Lr21* line was inoculated with *P. triticina* virulent race no change in the expression levels of Tc-*Lr21* transcripts were observed when compared to Thatcher.

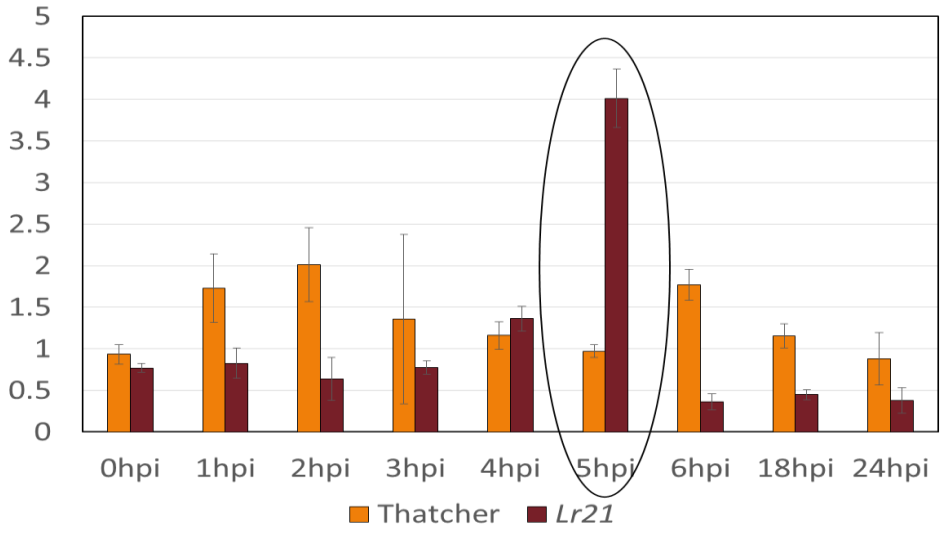

Figure 24. Real time-qPCR analysis of expression profiles of Tc-*Lr21* gene in wheat seedling leaves of susceptible cv. Thatcher and Tc-*Lr21* infected by wheat leaf rust fungus *P. triticina* avirulent race THBL at different time points.

The amount of transcript was normalized to GAPDH genes and was expressed as the percentage of the reference gene transcripts. Error bars represent the variation among three independent replications.

Discussion

Race-specific resistance has been studied as a source of resistance traits that might help in improving crop performance in field conditions (Parlevliet, 1983). In the present study, a continuum of layered defenses led to race-specific resistance in Tc-NIL's Tc-*Lr9* and Tc-*Lr21*. Highly visible hypersensitive flecks (;) were observed on Tc-*Lr9* leaves when inoculated with *P. triticina* indicating highly resistant reaction (race-specific resistance to races tested: THBL, MCDL and TNBJ). Fluorescent microscopic observations revealed that Tc-*Lr9* was associated with pre-haustorial resistance i.e., early abortion (no further growth and development) of infection units after appresorium at approximately 4 hpi was observed. Similar findings confirming pre-haustorial resistance by *Lr9* associated with strong callose deposition (Wang et al. 2013). However, in responses to *P. triticina* infection a hypersensitive response (HR) with cell death in infection units was observed as early as 30 mpi and with appresorium formation at approximately at 4 hpi and at 24 hpi and 48 hpi by light microscopic examination. Similar to previous studies Wang et al. 2007, and Orczyk et al. 2010 H_2O_2 were mainly detected in the cells surrounding the guard cells where appresoria was observed in clumps and in large quantities. Production and accumulation of ROS, such as hydrogen peroxide (H_2O_2), hydroxyl ions (OH^-) and superoxide anion (O2¡) at the penetration sites are often associated with early host defense responses (Thordal-Christensen et al. 1997). According to Wang et al. 2007, and Orczyk et al. 2010, Oxidative burst and ROS accumulation play a vital role in triggering resistance reaction pathways against rust fungi in wheat. In addition to cellular structures changes, we found that H_2O_2 and some defense-related genes also might contribute in preventing *P. triticina* infection of Tc-*Lr9*. Our results indicate hypersensitive induced genes *TaHIR1* was up-regulated at later time points 18 hpi and 24 hpi. However, *TaHIR2* gene was down regulated at early time point 6 hpi,

18 hpi and upregulated at 24 hpi. HIR genes might have indeed involved in H_2O_2 accumulation at later time points that is at 18 hpi and 24 hpi.

Despite the Tc-*Lr21* gene was cloned, very little information is available at molecular level regarding how early Tc-*Lr21* gene express and suppress the growth and development of the pathogen. Tc-*Lr21* was characterized through a map-based cloning method and it belongs to nucleotide binding site-leucine rich repeat (NBS-LRR) gene family. The NBS domain is believed to participate in signal transduction, LRR domain is thought to be involved in ligand binding and pathogen recognition (Young, 2000). NBS-LRR gene interacts in a gene-for-gene manner which involves HR confers a race-specific resistance. A typical characteristic of HR is local necrosis that occurs at and around the infection sites (Fen Liu et al. 2013, Heath 2000, Dangl and jones, 2001 and Park, 2005). Histo-chemical and Molecular pathogenicity of leaf rust in Tc-*Lr21* was studied by H_2O_2 accumulation and gene expression after leaf rust infection with susceptible cv. Thatcher and resistant line Tc-*Lr21* with both susceptible and resistance interactions. This represents an ideal situation for comparative differential gene expression patterns in Tc-*Lr21* gene. According to Feuillet et al. (2003), race-specific resistance gene Tc-*Lr21* contains a leucine-rich repeat domain (LRR) for recognition of pathogen effectors. Disease resistance and susceptibility are governed by the interaction of host receptors (which may be encoded by resistance genes) and pathogen elicitors. Hypersensitive cell death is the result of activation of host defense mechanisms which involves recognition of pathogen, and activates signal transduction cascade that may involve protein phosphorylation, ion fluxes, and reactive oxygen species (ROS), accumulation of antimicrobial compounds, cytoskeletons, salicylic acids, cysteine proteases and other signaling pathways (Fen Liu et al. 2013 and Manickavelu et al. 2010). In our study production of ROS like H_2O_2 was observed in Tc-*Lr21* as early as 5 hpi (at

the approximately appresorium formation and no haustorium is present) similar to the study of Orczyk et al. (2010) in resistant lines Tc-*Lr 9,* Tc-*Lr19* and Tc-*Lr26* H_2O_2 was observed at the site of appresorium formation. H_2O_2 accumulation might be induced due to the pressure generated by the appresorium, to penetrate in to substomatal chamber, as the mechanical stimulus can induce defense responses including an oxidative burst (Gus-Mayer et al. 1998) which is coincided with the upregulation of Tc-*Lr21* gene showed peak at 5 hpi in resistance interaction than the susceptible one. Similarly, our results agree with Abd EI-Aal et al. 2007, who amplified Tc-*Lr21* gene and upregulation was observed in both healthy and infected plants yet the expression was higher in resistant infected plant Giza 168 (Abd EI-Aal et al. 2007). The differential gene expression from our study might suggest the possible role of Tc-*Lr21* gene and recognition of effectors of *P. triticina* by an NB-LRR protein might be helping to trigger/ activate signaling pathways at early points (as early as 5 hpi). The present investigation showed that in Tc-*Lr21- P. triticina* interactions, cell death (HR) besides being an expression of Tc-*Lr21* gene before haustorium formation suggests that Tc-*Lr21- P. triticina* may also acts as a signal for PAMP triggered immunity (PTI) that is recognizing the pathogen early and activation of defense responses to suppress the pathogen rather than the ETI (effector triggered immunity) that is suppressing the pathogen after the formation of at least one haustorium by a pathogen (at approximately 24 hpi). According to Ferreira et al. (2006) and Orczyk et al. (2010) PTI would be commonly observed in non-host resistance mechanisms, however, there is a continuum between the resistance reactions of host pathogenesis to non-host and host interactions. The data obtained from this study provide opportunities to assess components of different resistance mechanisms and suggest that some previous assumptions about plant-pathogen interaction in the host and

non-host systems at early time points involving pre-and post-haustorial resistance should be revisited.

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CHAPTER FIVE: RAPID PROTOCOL FOR VISUALIZATION OF RUST FUNGI STRUCTURES USING FLUOROCHROME UVITEX 2B[2].

Abstract

Histological examination using fluorochromes is one of the standard methods for observation of microorganisms in tissues and other compartments. In the study of fungi, especially those that cannot be cultured in axenic media such as biotrophic fungi, histological examination of processes associated with the fungal growth, differentiation, infection and other cellular functions can lead to the better understanding of host-parasite interactions. Fluorescence microscopy coupled with Fluorochrome Uvitex 2B have been extensively utilized to study rust fungi structures and host-pathogen interactions. In this study, we report development of a rapid staining protocol of the rust fungus *Puccinia triticina* using fluorochrome Uvitex 2B. The newly developed rapid procedure was compared with a standard staining technique to observe *in planta* fungal infection structures development during the wheat – *Puccinia triticina* interaction. While significantly reducing the time for staining, the rapid protocol described here was equally efficient or better compared to standard procedure in detecting fungal infection structures using Uvitex 2B. In the rapid staining procedure, pre-heating of the stain increased efficiency to detect all the infection structures including haustoria with minimum highly reduced background noise from plant tissue. This staining process described here is simple and quick. It can be completed in four hours, which is of 6 times faster than the standard Uvitex 2B staining procedure.

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[2]The material in this chapter is reprinted from the article Dugyala, S., Borowicz, P., and Acevedo, M. 2015. Rapid protocol for visualization of rust fungi structures using fluorochrome Uvitex 2b. Plant Methods 11:54. © Dugyala et al. 2015. Sheshanka Dugyala's role in this manuscript was the conduction of all experiments and analyses and interpretation of microscopy data. Additionally, Sheshanka Dugyala wrote the manuscript and performed corrections suggested by the co-authors and the journal's reviewers.

Introduction

Histology is considered as one of the most appropriate technique to detect fungus and its infection structure development in plant and animal systems. The histological examination of fungal structure morphology and development during the infection process has offered valuable information to mycologists and plant pathologists for diagnosis, identification, classification of pathogenic microorganisms and their interaction with host and non-host plants specially when dealing with biotrophic fungi such as rust pathogens [1,2]. In recent years, a new interest in histology has been prompted by the availability of more powerful microscopes and the interest on connecting molecular and functional genomics discoveries to physical components. The fluorochromes Calcoflour White and Uvitex 2B and fluoresce microscopy have been extensively utilized to study rust fungi structures and host-pathogen interactions [3-14]. Both fluorochromes, Uvitex 2B and Calcoflour White, bind to the chitin present in the fungal body wall allowing fungal detection [15,16]. However, Calcoflour White fades away quickly when exposed to light, including its specific excitation wavelengths and stains plant structures containing cellulose making it difficult to differentiate fluorescence between the plant structures like trichomes, and fungal structures [14,17,18]. Therefore, Uvitex 2B has been more widely used to study rust fungi infection structures development in host and non-host interactions [1,12,19,20,21]. Currently used staining procedures are time consuming, and involve numerous chemical reagents including chloroform, methanol, lactophenol, ethanol, and NaOH. The procedure for fixation and staining of rust fungi as described by K. H. Kuck 1981 [23] has been the most widely used for staining rust fungi. This protocol requires 12-18 hours for fixing and clearing of specimens, depending on the stage of the plant development (adult or seedling) and staining requires an additional five hours. The length of the protocol and numerous steps has limited its applicability when dealing

with large number of specimens or when downstream molecular biology applications require intact cells and cell components such as nucleic acids.

The objective of the current study was to develop a rapid and less complex staining procedure that can be utilized to evaluate all infection structures in *Puccinia triticina* infection of wheat. We describe a rapid Uvitex 2B staining protocol that can be utilized for easy and comprehensive observation of the wheat leaf rust pathogen *Puccinia triticina* fungal structures *in planta* using wide-field fluorescence and structured illumination techniques. The newly developed rapid procedure was compared with the Uvitex 2B standard staining technique to observe *in planta* fungal infection structures development during wheat – *Puccinia triticina* interaction.

Materials and Methods

Plant material

Wheat cultivar Thatcher [26], susceptible to most of the known wheat leaf rust races was used in this study. Seedling were grown in flats in a rust-free greenhouse at (18 to 21^{o}C day, 16-18 ^{o}C night, 16h photoperiod) until second leaf stage was reached (primary leaf fully expanded).

Experimental design

The experimental design was a completely randomized design, with each cell containing three seedlings considered as an experimental unit replication. Three replications were used for microscopic observations at 2 hpi, 4 hpi, 6 hpi, 12 hpi, 18 hpi, 24 hpi, 48 hpi, 72 hpi, 96 hpi and 7 dpi for both staining methods (rapid and traditional staining methods).

Pathogen inoculations

A single spore isolate of the wheat leaf rust pathogen, *Puccinia triticina,* race THBL was used for inoculation. Fresh spores were utilized and urediniospores concentration was adjusted to

6.2 x 10^5 spores per ml in Soltrol 170 oil. Inoculations were done using a spray inoculator at secondary leaf stage. After inoculation, plants were incubated in a mist chamber with a 100% relative humidity (RH) for 16 h in dark and then transferred to a greenhouse compartment kept at 18-22^{o}C (day) and 18-20^{o}C (night) with a 16h photoperiod, and 80% RH.

Sample preparation

For all microscopic observations two 3-cm leaf pieces from the central portion of primary leaf were collected in a time course at 6 hpi, 24 hpi, 48 hpi, 72 hpi, 96 hpi and 7dpi. After cutting the leaf tissue all specimens were placed in a plastic centrifuge tube and placed in an ice bath until further processing.

Development of rapid staining protocol

To develop the rapid staining protocol using Uvitex 2B, specimens collected from 24 hpi were used as all the infection structures could be observed at this time point. Fixing and clearing was performed in a single step as previously described by Moldenhauer *et al.*., [12], with some modifications. In short, fixing and clearing involved placing each specimen in a 60mm glass petri dish containing 15mL Farmer's fixative (ethanol: acetic acid 3:1 v/v) and shake for 1 hr at 140 rpm on an orbital shaker at room temperature (approx. 21°C). Specimens were washed with deionized water twice for 10 min each. To determine the optimal conditions for rapid staining of specimens, multiple combination of pre-staining, staining and incubation time and temperature, were tested (Table 9). After staining, specimens were washed once with deionized water for 10 min and stored in 50% glycerol until specimens were mounted on microscopic slides.

Table 9. Combinations of staining temperature and pre-staining incubation time tested to determine best conditions to differentiate fungal structures when staining wheat leaf specimens inoculated with *P. triticina*.

Staining temperature	Room temperature	65° C
Pre-staining incubation time	0 min	0 min
	5 min	5 min
	10 min	10 min
	15 min	15 min
	30 min	30 min

Standard staining procedure

To determine if the new rapid protocol provided reliable data, equal or better than the previously described Uvitex 2B staining protocols, a second set of specimens from the experiment described above were stained using the standard protocol which is a modified version of Rohringer *et al..,* [26] as described by Moldenhauer *et al.* [12]. This protocol was used as the standard as it has been the most widely used procedure in previously published histological evaluation research using Uvitex 2B [3-14]. Briefly, infected leaves were fixed and cleared with lactophenol-ethanol or chloroform-methanol mostly by boiling method, and then washed with a series of different solutions including ethanol, NaoH, and water before and after staining the specimens.

Microscopic observations

Three primary leaves sections (2 cm each) from each of the three replicates were collected and six slides (two slides per each primary leaf) were mounted for microscopic observations. A total of 10-15 infection units were observed per each slide per time point and images of representative infection sites were acquired. Specimens were examined under wide-field fluorescence using an Zeiss Axio Imager M2 research epifluorescence upright microscope with an excitation filter BP379-401; chromatic beam splitter FT 420; emission 435-485; using AxioVision rel. 4.8 software. All observations were carried at 5X, 20X and 40X. For confocal

microscopy and haustoria observation, specimens were observed using Zeiss Axio Observer Z1 inverted microscope with LSM700 laser scanning unit, 405 solid state laser at 40x magnification. Staining procedures were evaluated for their efficiency in visualizing pathogen infection structures including spore, germ tube, appresorium, sub-stomatal vesicle (SSV), haustorial mother cell (HMC) and haustorium; and background noise from staining host structures.

Results

Rapid protocol development and comparison to standard protocol

Infected leaves of susceptible wheat cultivar Thatcher were sampled at various time points, stained using both staining methods (rapid and standard), and observed under the fluorescence microscope. In the rapid staining procedure, clearing and fixing steps were combined into one single step by replacing chloroform-methanol and lactophenol-ethanol, with ethanol: acetic acid (3:1 v/v) (Figure 27a,b). Since Farmer's fixative only requires a short period of time to clear and fix specimens, the 18-24h period required for clearing and fixing in the standard procedure was reduced to just one hour. By using Farmer's fixative instead of lactophenol-ethanol no additional ethanol, 0.1N NaOH or water washing steps were required as recommended in the standard Uvitex 2B method [8-14,24].

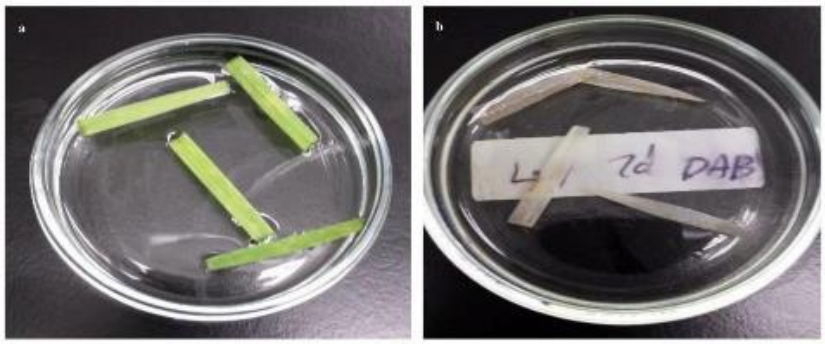

Figure 25. Conditions and characteristics for clearing and fixing of specimens.

a) Wheat cultivar Thatcher leaf tissue collected and placed directly into Farmer's fixative immediately after collection. **b)** Fixed and cleared specimens after 60 min in Farmer's fixative.

Soaking of specimens in 0.1M Tris-HCl (pH 8.5) before staining step was found to be necessary for effective staining. Specimens were not stained when not soaked in Tris-HCl or poorly stained, when soaked for only 5 min irrespective of staining temperature, (Figure 28a). Specimens were stained uniformly when soaked in Tris-HCl for at least 10 min (Figure 28b). No apparent staining advantage was noticed with increase pre-stain soaking time (15 or 30 min) compared to 10 min. Overall, specimens stained with pre-heated 0.3% Uvitex 2B appeared bright and haustoria were clearly visible compared to staining solution at room temperature. In addition, a reduction of non-specific staining was achieved. Based on the above results, fixing and clearing for only 1h, pre-stain soaking in 0.1M Tris-HCl for 10 min and staining with pre-heated 0.3% Uvitex 2B were identified as optimum conditions for visualization of fungal structures *in planta* using fluorescence microscopy.

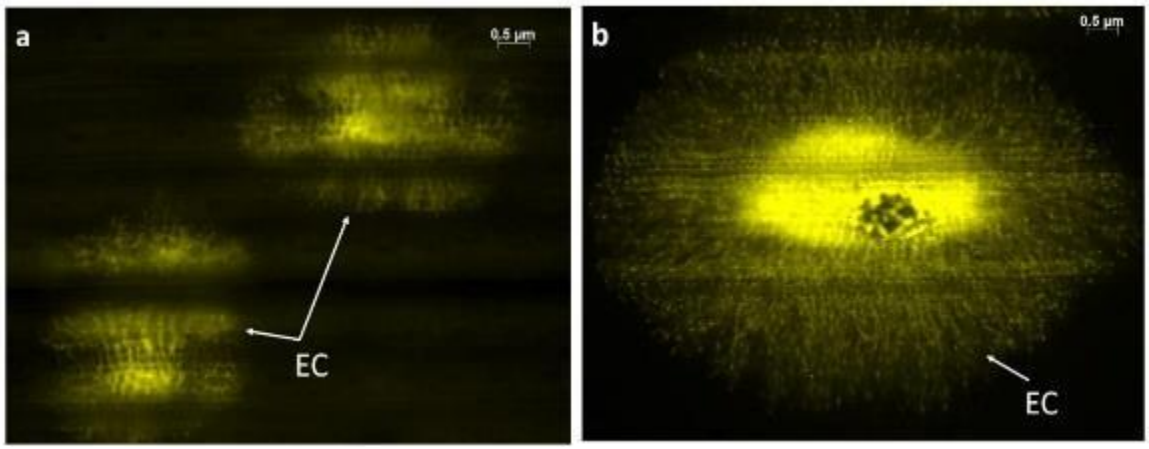

Figure 26. Comparison of pre-staining incubation time.

Leaves of seedling stage plants of cultivar Thatcher infected by *Puccinia triticina* were stained with fluorochrome Uvitex 2B and examined under an Axio Imager M2 Zeiss Research epifluorescence microscope. **a)** Under-stained *P. triticina* colony in leaf tissue treated with a 5 min pre-staining incubation. **b)** Brighter *P. triticina* colony after a 10 min pre-staining treatment in 0.1M Tris-HCl. Established colony (EC).

Using both these methods (standard and rapid), evaluation of fungal structures on Thatcher seedling plants after inoculation with *P. triticina* race THBL uredinospores was

possible. Spore germination and germ tube was observed within 2h after spraying of inoculum onto seedling plants leaf surface (Figure 3A-H). Germinating urediniospores in many cases had two germ tubes but only one of the germ tubes differentiated into an appresorium. (Figure 29a). Germ tube branching was observed as previously described for *P. triticina* [25]. At 4 hpi, appresorium with 3-4 lobes were observed in the stomata on the leaf surface (Figure 29b) After the appresorium was formed, other infection structures including infection peg, sub-stomatal vesicle, and infection hyphae were observed at 12 hpi (Figure 29c). Haustorial mother cells (HMC) were observed by 18 hpi (Figure 29d). In later time points (48 hpi, 72 hpi and 96 hpi) an increase number of HMC, and further colony development was observed (Figure 29e-g). Finally, by seven dpi, completely established colonies with sporulation were observed (Figure 29h). Matured and newly formed spores were observed in established colonies (Figure 29h). Despite not able to observe haustoria under wide-field fluorescence microscope at any of the tested magnifications, haustoria were clearly observed inside the plant cell by 20 hpi, with laser scanning confocal microscope at 40 X magnification (Figure 30a,b).

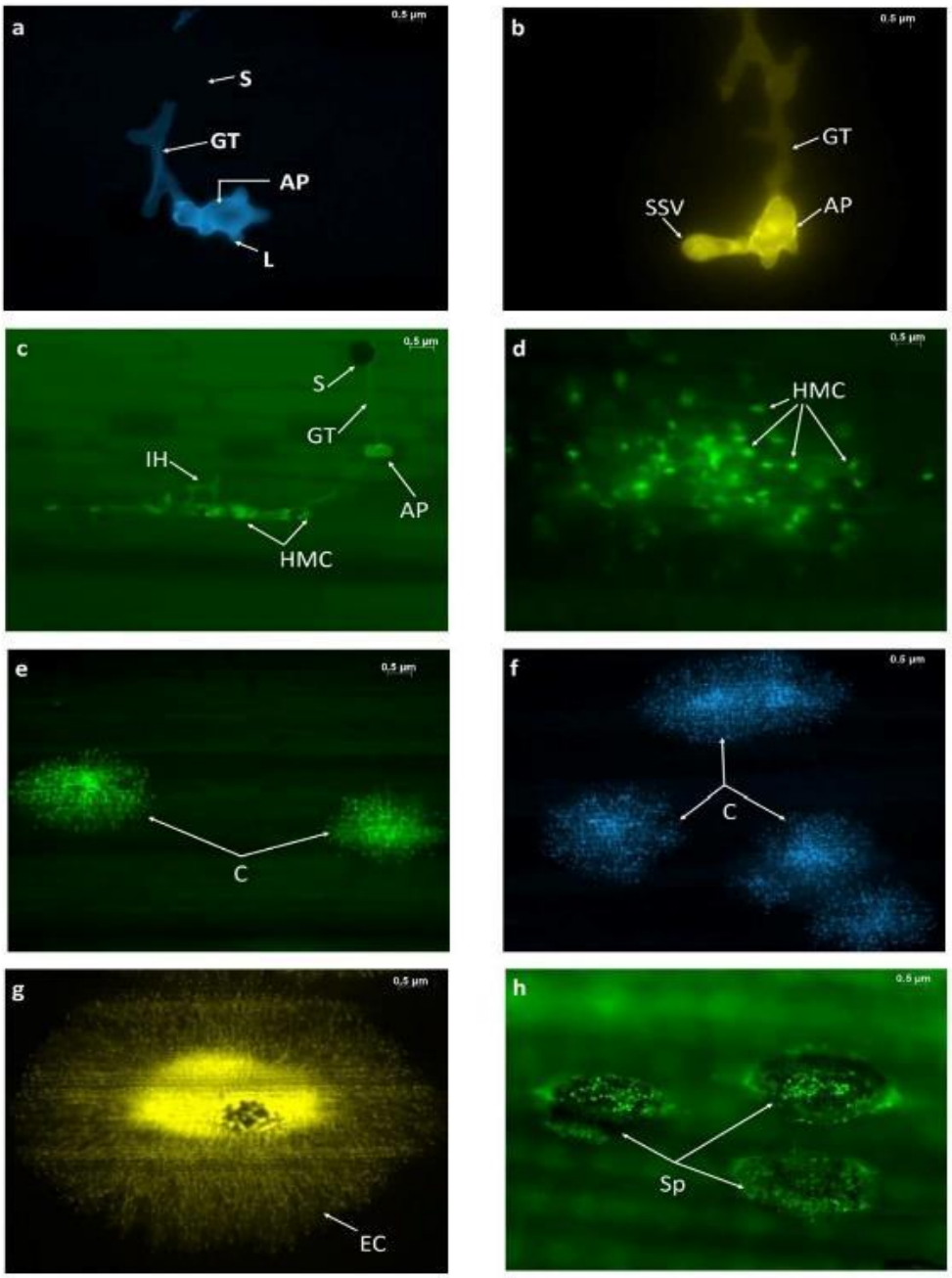

Figure 27. *P. triticina* infection on susceptible wheat cultivar Thatcher.

Seedling stage plant specimens examined under an Axio Imager M2 Zeiss Research epifluorescence microscope (**a-h**). **a)** Germinated spore (S) 4 hpi, with branching germ tube (GT) and appresorium (AP) with lobes (L). **b)** At 12 hpi, infection peg formed from appresorium and sub-stomatal vesicle (SSV) formation from infection peg. **c)** At 12 hpi, Formation of infection hyphae (IH) from infection peg. **d)** At 18 hpi, haustorial mother cell (HMC) produced a penetration peg that perforated through mesophyll cell wall and formed a haustorium in the host cell. **e-f)** At 24 hpi-96 hpi, increase number of haustorial mother cells, and colony development (C). **g-h)** At 7 dpi, established colony (EC) and sporulation (SP).

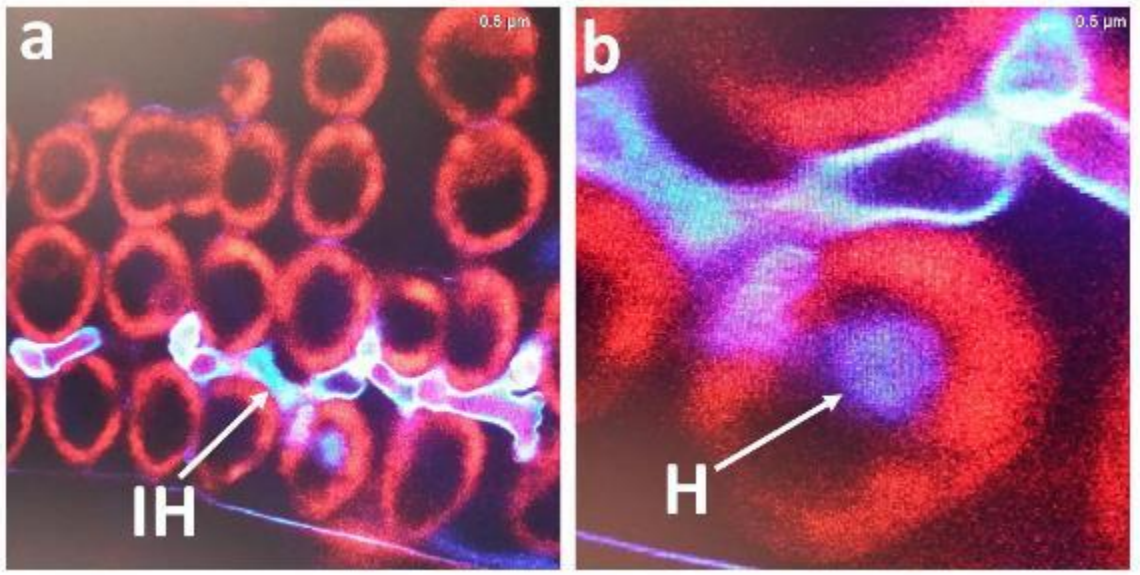

Figure 28. Confocal microscopy of haustoria from wheat-*P. triticina* infected tissue.

Seedling stage plant specimens examined under Laser scanning Alexa Fluor 488 confocal microscope (a-b). a) At 20 hpi, formation of intercellular primary infection hyphae (IH), differentiated in to haustorial mother cells (HMC) once in contact with mesophyll cells of host. b) Haustorial mother cells penetrated through the host mesophyll cells wall to form haustorium (H).

In a small preliminary experiment, we used the rapid Uvitex 2B to stain *P. triticina* inoculated wheat leaf samples prepared for Laser Capture Microdissection and RNA extraction of host and pathogen from 500 dissected cells. Using this method, we were able to successfully identify infected cells (Figure31a,b) for excision. Total RNA was then extracted from the excised infected cells and used in the amplification of the *P. triticina* ubiquitin gene. The RNA amplification threshold cycles for Ubiquitin of the LCM specimens were comparable to those obtained from diluted RNA specimens extracted from full wheat leaf tissue without staining or LCM preparation (Acevedo et al. unpublished).

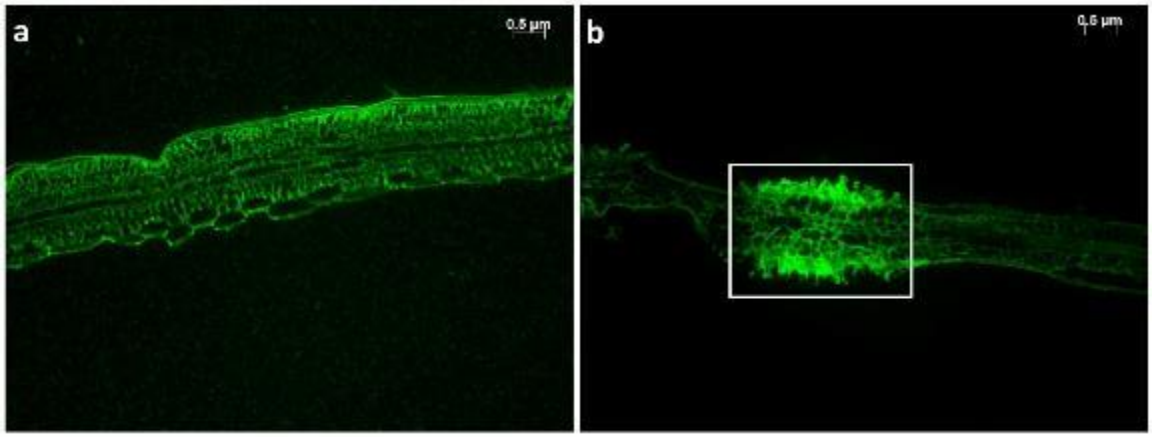

Figure 29. Sample preparation for Laser Capture Microdissection of infected wheat cells.

Wheat leaf specimens examined under an Axio Imager M2 Research epifluorescence microscope (a-b). a) Longitudinal section of non-inoculated wheat leaf stained with Uvitex 2B. b) Longitudinal section of wheat leaf inoculated with *P. triticina*. Within box, infection site with uredinium was excised via LCM and collected for RNA extraction of host and pathogen cells.

Conclusions

We have developed a rapid and effective way to stain wheat leaf tissue infected with the fungal pathogen *P. triticina* and were able to clearly observe and distinguish all fungal infection structures *in planta* in a time course experiment. In the newly developed rapid Uvitex 2B protocol there is only a single step involved for fixing and clearing the specimen and it reduced the time from 18-24 hours to only 1 hour. By pre-heating the fluorochrome Uvitex 2B a brighter staining of fungal structures and better contrast against plant cell was obtained.

Currently developed rapid staining protocol has several advantages over the standard staining protocol that has been used to study fungal infection process *in planta*. First, staining the specimens using this rapid staining procedure is fast and simple, fixation and staining can be completed within 1 and 3 hours respectively. Second, the pre-heating of the Uvitex 2B before the staining step allows for better detection of fungal structures including haustoria (when coupled with confocal microscopy) and provides a clear differentiation between host and fungal

structures. Third, with this rapid staining procedure the number of chemical reagents is reduced which results in a lower cost per specimen. Moreover, due to the short time required from collection to observation of specimens and rapid specimen fixation step, the rapid Uvitex 2B staining protocol is suitable for use in the preparation of samples for plant-cell isolations maintaining nucleic acid integrity. The ability to amplify RNA from excised cells from the Uvitex 2B stained samples demonstrates that the rapid staining protocol does not affect quality of RNA making this protocol suitable for specific cell gene expression studies. Furthermore, the rapid staining procedure has been successfully applied to other rust pathogen-host and non-host interaction including *Puccinia graminis* f.sp. *tritici- Hordeoum vulgare, P. triticina – Triticum turguidum,* P. *triticina- H. vulgare* and *Puccinia coronata* f. sp. *avenae- Avena sativa* (data not shown). Future work will determine if the rapid staining protocols can be utilized in other pathosystems outside of the cereal rusts.

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APPENDIX A. COMPARISON OF *LR* GENES AT DIFFERENT TIME POINTS TO INFECTION STRUCTURE FORMATION

Table A1. Comparison of *Lr* genes at different time points to infection structure formation with race THBL of *P. triticina* at adult-plant stage.

Cultivar	Treatment	Infection structures					Colony	
		SP	GT	AP	SSV	TC	Aborted	Established
Thatcher	Non-Inoc	0	0	0	0	0	0	0
	Mock-Inoc	0	0	0	0	0	0	0
	0 hpi	1	0	0	0	0	0	0
	6 hpi	1	1	0	0	0	0	1
	12 hpi	1	1	1	0	0	0	1
	24 hpi	1	1	1	1	1	0	1
	48 hpi	1	1	1	1	1	0	1
	72 hpi	1	1	1	1	1	0	1
	96 hpi	1	1	1	1	1	0	1
	7dpi	1	1	1	1	1	0	1
Tc-*Lr9*	Non-Inoc	0	0	0	0	0	0	0
	Mock-Inoc	0	0	0	0	0	0	0
	0 hpi	1	0	0	0	0	0	0
	6 hpi	1	1	0	0	0	0	1
	12 hpi	1	1	0	0	0	0	1
	24 hpi	1	1	1	0	0	1	1
	48 hpi	1	1	1	1	0	1	0
	72 hpi	1	1	1	1	0	1	0
	96 hpi	1	1	1	1	0	1	0
	7dpi	1	1	1	1	0	1	0
Tc-*Lr21*	Non-Inoc	0	0	0	0	0	0	0
	Mock-Inoc	0	0	0	0	0	0	0
	0 hpi	1	0	0	0	0	0	0
	6 hpi	1	1	0	0	0	0	1
	12 hpi	1	1	0	0	0	0	1
	24 hpi	1	1	1	0	0	0	1
	48 hpi	1	1	1	0	0	0	1
	72 hpi	1	1	1	1	0	0	1
	96 hpi	1	1	1	1	1	0	1
	7dpi	1	1	1	1	1	0	1
Tc-*Lr34*	Non-Inoc	0	0	0	0	0	0	0
	Mock-Inoc	0	0	0	0	0	0	0
	0 hpi	1	0	0	0	0	0	0
	6 hpi	1	1	0	0	0	0	0

Table A1. Comparison of *Lr* genes at different time points to infection structure formation with race THBL of *P. triticina* at adult-plant stage. (Continued).

Cultivar	Treatment	Infection structures					Colony	
		SP	GT	AP	SSV	TC	Aborted	Established
	12 hpi	1	1	0	0	0	0	0
	24 hpi	1	1	1	0	0	0	1
	48 hpi	1	1	1	1	0	1	1
	72 hpi	1	1	1	1	1	1	1
	96 hpi	1	1	1	1	1	1	1
	7dpi	1	1	1	1	1	1	1
Tc-*Lr35*	Non-Inoc	0	0	0	0	0	0	0
	Mock-Inoc	0	0	0	0	0	0	0
	0 hpi	1	0	0	0	0	0	0
	6 hpi	1	1	0	0	0	0	1
	12 hpi	1	1	0	0	0	0	1
	24 hpi	1	1	1	1	0	0	1
	48 hpi	1	1	1	1	0	0	1
	72 hpi	1	1	1	1	0	0	1
	96 hpi	1	1	1	1	1	0	1
	7dpi	1	1	1	1	1	0	1
Harrington	Non-Inoc	0	0	0	0	0	0	0
	Mock-Inoc	0	0	0	0	0	0	0
	0 hpi	1	0	0	0	0	0	0
	6 hpi	1	1	0	0	0	0	1
	12 hpi	1	1	1	0	0	0	1
	24 hpi	1	1	1	0	0	0	1
	48 hpi	1	1	1	1	0	1	0
	72 hpi	1	1	1	1	0	1	0
	96 hpi	1	1	1	1	0	1	0
	7dpi	1	1	1	1	0	1	0
Q21681	Non-Inoc	0	0	0	0	0	0	0
	Mock-Inoc	0	0	0	0	0	0	0
	0 hpi	1	0	0	0	0	0	0
	6 hpi	1	1	0	0	0	1	0
	12 hpi	1	1	1	0	0	1	0
	24 hpi	1	1	1	0	0	1	0
	48 hpi	1	1	1	1	0	1	0
	72 hpi	1	1	1	1	0	1	0
	96 hpi	1	1	1	1	0	1	0
	7dpi	1	1	1	1	0	1	0

Table A2. Comparison of *Lr* genes at different time points to infection structure formation with race THBL of *P. triticina* at seedling stage.

Cultivar	Treatment	Infection structures					Colony	
		SP	GT	AP	SSV	TC	Aborted	Established
Thatcher	Non-Inoc	0	0	0	0	0	0	0
	Mock-Inoc	0	0	0	0	0	0	0
	0 hpi	1	0	0	0	0	0	0
	6 hpi	1	1	0	0	0	0	1
	12 hpi	1	1	1	0	0	0	1
	24 hpi	1	1	1	1	1	0	1
	48 hpi	1	1	1	1	1	0	1
	72 hpi	1	1	1	1	1	0	1
	96 hpi	1	1	1	1	1	0	1
	7dpi	1	1	1	1	1	0	1
Tc-*Lr9*	Non-Inoc	0	0	0	0	0	0	0
	Mock-Inoc	0	0	0	0	0	0	0
	0 hpi	1	0	0	0	0	0	0
	6 hpi	1	1	0	0	0	0	0
	12 hpi	1	1	0	0	0	0	0
	24 hpi	1	1	1	0	0	1	0
	48 hpi	1	1	1	1	0	1	0
	72 hpi	1	1	1	1	0	1	0
	96 hpi	1	1	1	1	0	1	0
	7dpi	1	1	1	1	0	1	0
Tc-*Lr21*	Non-Inoc	0	0	0	0	0	0	0
	Mock-Inoc	0	0	0	0	0	0	0
	0 hpi	1	0	0	0	0	0	0
	6 hpi	1	1	0	0	0	0	1
	12 hpi	1	1	0	0	0	0	1
	24 hpi	1	1	1	0	0	0	1
	48 hpi	1	1	1	0	0	1	1
	72 hpi	1	1	1	1	0	0	1
	96 hpi	1	1	1	1	1	0	1
	7dpi	1	1	1	1	1	0	1
Tc-*Lr34*	Non-Inoc	0	0	0	0	0	0	0
	Mock-Inoc	0	0	0	0	0	0	0
	0 hpi	1	0	0	0	0	0	0
	6 hpi	1	1	0	0	0	0	0

Table A2. Comparison of *Lr* genes at different time points to infection structure formation with race THBL of *P. triticina* at seedling stage. (Continued).

Cultivar	Treatment	Infection structures					Colony	
		SP	GT	AP	SSV	TC	Aborted	Established
	12 hpi	1	1	0	0	0	0	0
	24 hpi	1	1	1	0	0	0	1
	48 hpi	1	1	1	1	0	0	1
	72 hpi	1	1	1	1	1	0	1
	96 hpi	1	1	1	1	1	0	1
	7dpi	1	1	1	1	1	0	1
Tc-*Lr35*	Non-Inoc	0	0	0	0	0	0	0
	Mock-Inoc	0	0	0	0	0	0	0
	0 hpi	1	0	0	0	0	0	0
	6 hpi	1	1	0	0	0	0	1
	12 hpi	1	1	0	0	0	0	1
	24 hpi	1	1	1	1	0	0	1
	48 hpi	1	1	1	1	0	0	1
	72 hpi	1	1	1	1	0	0	1
	96 hpi	1	1	1	1	1	0	1
	7dpi	1	1	1	1	1	0	1
Harrington	Non-Inoc	0	0	0	0	0	0	0
	Mock-Inoc	0	0	0	0	0	0	0
	0 hpi	1	0	0	0	0	0	0
	6 hpi	1	1	0	0	0	0	1
	12 hpi	1	1	1	0	0	0	1
	24 hpi	1	1	1	0	0	0	1
	48 hpi	1	1	1	1	0	0	1
	72 hpi	1	1	1	1	0	0	1
	96 hpi	1	1	1	1	0	0	1
	7dpi	1	1	1	1	0	0	1
Q21681	Non-Inoc	0	0	0	0	0	0	0
	Mock-Inoc	0	0	0	0	0	0	0
	0 hpi	1	0	0	0	0	0	0
	6 hpi	1	1	0	0	0	1	0
	12 hpi	1	1	1	0	0	1	0
	24 hpi	1	1	1	0	0	1	0
	48 hpi	1	1	1	1	0	1	0
	72 hpi	1	1	1	1	0	1	0
	96 hpi	1	1	1	1	0	1	0
	7dpi	1	1	1	1	0	1	0

Table A3. Comparison of *Lr* genes at different time points to infection structure formation with race MCDL of *Puccinia triticina* at seedling stage.

Cultivar	Treatment	Infection structures					Colony	
		SP	GT	AP	SSV	TC	Aborted	Established
Thatcher	Non-Inoc	0	0	0	0	0	0	0
	Mock-Inoc	0	0	0	0	0	0	0
	0 hpi	1	0	0	0	0	0	0
	6 hpi	1	1	0	0	0	0	1
	12 hpi	1	1	1	0	0	0	1
	24 hpi	1	1	1	1	1	0	1
	48 hpi	1	1	1	1	1	0	1
	72 hpi	1	1	1	1	1	0	1
	96 hpi	1	1	1	1	1	0	1
	7dpi	1	1	1	1	1	0	1
Tc-*Lr9*	Non-Inoc	0	0	0	0	0	0	0
	Mock-Inoc	0	0	0	0	0	0	0
	0 hpi	1	0	0	0	0	0	0
	6 hpi	1	1	0	0	0	0	0
	12 hpi	1	1	0	0	0	0	0
	24 hpi	1	1	1	0	0	1	0
	48 hpi	1	1	1	1	0	1	0
	72 hpi	1	1	1	1	0	1	0
	96 hpi	1	1	1	1	0	1	0
	7dpi	1	1	1	1	0	1	0
Tc-*Lr21*	Non-Inoc	0	0	0	0	0	0	0
	Mock-Inoc	0	0	0	0	0	0	0
	0 hpi	1	0	0	0	0	0	0
	6 hpi	1	1	0	0	0	0	1
	12 hpi	1	1	0	0	0	0	1
	24 hpi	1	1	1	0	0	0	1
	48 hpi	1	1	1	0	0	0	1
	72 hpi	1	1	1	1	0	0	1
	96 hpi	1	1	1	1	1	0	1
	7dpi	1	1	1	1	1	0	1
Tc-*Lr34*	Non-Inoc	0	0	0	0	0	0	0
	Mock-Inoc	0	0	0	0	0	0	0
	0 hpi	1	0	0	0	0	0	0
	6 hpi	1	1	0	0	0	0	0

Table A3. Comparison of *Lr* genes at different time points to infection structure formation with race MCDL of *Puccinia triticina* at seedling stage. (Continued).

Cultivar	Treatment	Infection structures					Colony	
		SP	GT	AP	SSV	TC	Aborted	Established
	12 hpi	1	1	0	0	0	0	0
	24 hpi	1	1	1	0	0	0	1
	48 hpi	1	1	1	1	0	0	1
	72 hpi	1	1	1	1	1	0	1
	96 hpi	1	1	1	1	1	0	1
	7dpi	1	1	1	1	1	0	1
Tc-*Lr35*	Non-Inoc	0	0	0	0	0	0	0
	Mock-Inoc	0	0	0	0	0	0	0
	0 hpi	1	0	0	0	0	0	0
	6 hpi	1	1	0	0	0	0	1
	12 hpi	1	1	0	0	0	0	1
	24 hpi	1	1	1	1	0	0	1
	48 hpi	1	1	1	1	0	0	1
	72 hpi	1	1	1	1	0	0	1
	96 hpi	1	1	1	1	1	0	1
	7dpi	1	1	1	1	1	0	1
Harrington	Non-Inoc	0	0	0	0	0	0	0
	Mock-Inoc	0	0	0	0	0	0	0
	0 hpi	1	0	0	0	0	0	0
	6 hpi	1	1	0	0	0	0	1
	12 hpi	1	1	1	0	0	0	1
	24 hpi	1	1	1	0	0	0	1
	48 hpi	1	1	1	1	0	1	0
	72 hpi	1	1	1	1	0	1	0
	96 hpi	1	1	1	1	0	1	0
	7dpi	1	1	1	1	0	1	0
Q21681	Non-Inoc	0	0	0	0	0	0	0
	Mock-Inoc	0	0	0	0	0	0	0
	0 hpi	1	0	0	0	0	0	0
	6 hpi	1	1	0	0	0	0	0
	12 hpi	1	1	1	0	0	0	0
	24 hpi	1	1	1	0	0	1	0
	48 hpi	1	1	1	1	0	1	0
	72 hpi	1	1	1	1	0	1	0
	96 hpi	1	1	1	1	0	1	0
	7dpi	1	1	1	1	0	1	0