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Tracking evolutionary dynamics within the fungal wheat pathogen *Zymoseptoria tritici*

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Résumé

Les champignons pathogènes sont de nature très diverse et peuvent poser de graves risques par le biais d'infections simples ou mixtes à la production alimentaire mondiale. Le contrôle des cultures est basé sur l'utilisation de produits chimiques synthétiques ainsi que la sélection de plantes résistantes. Cependant, les pathogènes fongiques des plantes surmontent rapidement les deux stratégies. Lors de co-infections, les phytopathogènes peuvent augmenter la sévérité de la maladie et modifier l'évolution de la virulence des génotypes co-infectants. Par conséquent, les outils de surveillance permettant une meilleure détection des agents pathogènes des cultures ainsi que la compréhension de l'impact des événements de co-infection sur la gravité de la maladie ne sont pas toujours efficace. De plus, nous manquons encore d'informations sur la base génétique responsable de l'évolution de la virulence et de la résistance aux fongicides au fil du temps. Dans cette thèse de doctorat, J'ai concentrés sur le développement d'un outil de surveillance pour tracer l'évolution de la virulence et de la résistance aux fongicides chez le pathogène fongique du blé *Zymoseptoria tritici* à l'aide de différents outils moléculaires et génomiques. Ici, j'ai développé un outils de génotypage pour multiplexer plusieurs locus ciblant les gènes de virulence et de résistance aux fongicides, et des marqueurs sélectionnés au hasard à l'échelle du génome. Plus d'une centaine de types d'échantillons ont été utilisés pour évaluer la bonne performances de mon outil. Cela a ensuite permis une amplification précise de tous les locus conçus et a bien fonctionné quel que soit le type d'échantillon. J'ai également exploré les conséquences d'interactions mixtes à l'intérieur et à l'extérieur de la plante hôte en utilisant un échantillon de grande taille pour fournir une image plus approfondie des conséquences de ces interactions sur la gravité de la maladie de plante ainsi que sur le changement de virulence et de croissance. J'ai constaté que la cinétique de croissance et la virulence dans les interactions simples s'écartaient de celles observées lors des interactions mixtes, indiquant une preuve d'exclusion compétitive entre les souches conspécifiques. De plus, les résultats étaient divergents à l'intérieur et à l'extérieur de la plante hôte, ce qui indique que le système immunitaire de la plante pourrait jouer un rôle clé dans la modulation des conséquences de l'interaction mixte à l'intérieur de la plante et ajoute une complexité significative. Enfin, j'ai utilisé un grand jeux de donnée de la population mondiale de *Z.tritici* pour étudier les éléments transposables

situés à proximité d'importants locus de gènes de virulence et de résistance aux fongicides permettant de comprendre l'histoire évolutive du pathogène dans le monde ainsi que d'établir de meilleures stratégies de protection de cultures. J'ai identifié un nombre considérable d'éléments transposables appartenant à différentes familles et superfamilles. J'ai également détecté des insertions récentes représentées par des insertions uniques. Dans l'ensemble, cette thèse de doctorat permet le traçage des génotypes de *Z.tritici* nouvellement évolués et de comprendre la base génétique médiant leur évolution. En outre, fournir des informations sur les conséquences des interactions mixtes sur la gravité de la maladie et sur la manière dont elles peuvent façonner le changement de virulence et la capacité de croissance des champignons phytopathogènes.

Mots clés : champignon, plante, blé, *Zymoseptoria tritici*, génotypage, élément transposable, interactions microbiennes.

Summary

Plant pathogenic fungi are very widespread and can pose severe risks to global food production through single as well as mixed infection. Crop control is based on the use of synthetic chemicals and plant resistance breeding. However, fungal plant pathogens quickly overcome both strategies. During co-infections, plant pathogens can increase disease severity and change co-infecting genotypes virulence evolution. Hence, monitoring tools improving crop pathogen detection as well as understanding the impact of co-infection events on disease severity are lacking. Additionally, we still lack information on the genetic basis shaping virulence and fungicide resistance evolution over time. In this PhD thesis I focused on developing monitoring tool to track virulence and fungicide resistance evolution in the fungal wheat pathogen *Zymoseptoria tritici* using different molecular and genomic tools. Here I designed a microfluidics-based amplicon sequencing assay to multiplex several loci targeting virulence and fungicide resistance genes, and randomly selected genome-wide markers. More than hundred types of samples were used to assess the performance of our assay. This later allowed an accurate amplification of all designed loci and performed well regardless of the sample type. I also explored the outcomes of mixed interactions within and outside the plant host by using a large sample size to provide a deeper picture about the consequences of such interactions on disease severity as well as virulence and growth effects. I found that the growth kinetics and virulence in single interactions deviated from those seen during mixed interactions indicating competitive exclusion between conspecific strains. Additionally, the outcomes were divergent within and outside the plant host indicating that the plant immune system plays a key role in shaping the within-plant interaction outcomes and adds significant complexity. Finally, I used a data set of worldwide populations of *Z. tritici* to investigate transposable elements (TEs) located nearby important virulence and fungicide resistance genes allowing to retrace the evolutionary history of the pathogen worldwide as well as a better crop protection strategies. I identified a considerable number of TEs belonging to different families and superfamilies, I also detected recent insertions represented by unique insertions called singletons. Overall, this PhD thesis allows monitoring newly evolved *Z. tritici* genotypes and understanding the genetic basis mediating their adaptation to their hosts and environment. Besides, the thesis provides information about the

consequences of mixed interactions on disease severity and how these can shape change in virulence and growth of plant pathogens.

Key words: fungus, plant, wheat, *Zymoseptoria tritici*, genotyping, transposable element, microbial interactions.

Glossary

Accessory chromosome	They are excess chromosomes resulting from unsuccessful meiotic divisions and are found in some but not all individuals of a population. They are not essential chromosomes to the survival of the organism.
Appressoria	It is a specialized cell specific of many fungal plant pathogens that is used to infect host plants.
Blastospores	A vegetative fungal cell produced by budding.
Biotrophic pathogen	Pathogens which require living plant for their survival and infection.
Chlamydospore	They are solitary, and relatively thick-walled asexual spores.
Core chromosome	They are found in all individuals of a population and are essential chromosomes to the survival of the organism.
Effector protein	They are microbial virulence molecules that are secreted into the host and mediate the outcome of plant–microbe interactions.
Genome wide association studies (*GWAS*)	A research approach involves scanning genetic markers across the genomes of many people to identify genetic variations associated with disease.
Hemebiotrophic pathogen	They require living plant tissue to survive and complete their infection cycle. They show distinct phases of their life cycle: an early

	asymptomatic biotrophic phase and a late symptomatic necrotrophic phase.
Maximum specific growth rate (*μmax*)	The rate of increase of biomass of a microbe per unit of biomass concentration. The maximum specific growth rate of a microbe is assumed to be fairly constant.
Necrotrophic pathogen	They feed on the dead host tissues that they kill before or during colonization resulting in extensive necrosis.
Repeat-Induced Point Mutation (*RIP*)	A genome defense in fungi which consists of hypermutating repetitive DNA to limit and suppress the accumulation of transposable elements.
PLACL	Percentage of leaf area covered by lesions
Pycnidiospores	They are a common asexual spore among ascomycetes. They are formed from the of asexual fruiting bodies (pycnidia).
Single nucleotide polymorphism (*SNP*)	It is a genetic variation of a single nucleotide at a specific position in the genome.
Transposable element (*TE*)	They are DNA sequences that are able to change their position within a genome.

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Diverse sets of transposable elements contribute to the rapid sequence evolution of virulence and fungicide resistance loci in the fungal wheat pathogen Zymoseptoria tritici

General Introduction

Emerging pathogens can cause a new disease, significantly increase the incidence of a current disease, or introduce a disease outside of its place or origin to a new geographical areas (Fones et al. 2020). Pathogenic fungi have been responsible for emerging diseases in plants and animals (Nobile and Johnson 2015; Arie 2019). Fungi are responsible for about 30% of emerging diseases in plants and pose a significant risk to food security worldwide (Anderson et al. 2004). Normally, crop control relies on resistance breeding and the application of chemical compounds such as fungicide (Nelson et al. 2018; Corkley et al. 2022). However, such control strategies are not effective to fight against newly emerging fungi. Therefore, identifying the factors causing the emergence of new fungal diseases would be crucial for disease management. There are a number of ways in which diseases emerge; the pathogen can be introduced to a new area where its host is present by physical transportation (Anderson et al. 2004) or it may be introduced more gradually through the development of newly permissive abiotic factors, such as climate (Bebber et al. 2013). The most dramatic historical example is the epidemic caused by *Phytophthora infestans* on cultivated potato in Ireland in the 1840s (Birch and Whisson 2001). The Irish Potato Famine caused mass emigration and more than 1 million deaths from famine-related diseases (Giraud et al. 2010). The pathogen responsible for late blight of potato *P.infestans* spread from its center of origin in Mexico through North America and then to Europe in 1845 (Yoshida et al. 2013). The introduction of *P. infestans* to Europe in 1845 is a key example of an emerging pathogen: the pathogen was newly established in Europe as its initial host, the potato, was introduced to the region 300 years previously. Once established in Europe, the potatoes did not carry any resistance (R) genes against newly evolved *P. infestans* genotypes (Yoshida et al. 2013).That strain was the only genotype from the *P. infestans* species (Yoshida et al. 2013) but today multiple genotypes matching several resistance (R) genes on potato have been recognized in Europe, North America, and Australia (Fry 2008; Aguilera-Galvez et al. 2018; Elnahal et al. 2020). Additionally, fungi commonly undergo genetic recombination, hybridization, or horizontal gene transfer (Hastings 1992; Mehrabi et al. 2011; Richards et al. 2011). Such evolutionary forces cause the emergence of newly evolved genotypes but also allowing formation of genetic variability that may underlie variable virulence leading to the generation

of diverse genotypes enabling infection or co-infection of new host, or resistance to disease control measures. Hence, to facilitate effective management of plant disease it would be crucial to effectively detect newly evolved genotypes, understanding the evolutionary process involved as well as investigating their possible interactions within plant hosts.

Genetic basis of plant-pathogen interactions during infection

Plant diseases cause of 10–15% of the world's major crop losses, resulting in huge economic losses (Chatterjee et al. 2016; Food and Agriculture Organization of the United Nations (FAO) 2019).Approximately 30 percent of all crop diseases are caused by fungi (Jain et al. 2019). Fungal plant pathogen can infect a wide range of plant hosts (Bai and Shaner 2004; Kolmer et al. 2009; Li et al. 2020; Sutacha et al. 2022) and they are very diverse and primarily belonging to the phyla of *Ascomycota* and *Basidiomycota* (Doehlemann et al. 2017). To spread infections, fungal spores are usually dispersed by wind or water and are able to attach firmly to different hosts by producing adhesive extracellular matrix (Doehlemann et al. 2017). For example, spores of *M. oryzae* to attach firmly to their host form a spore-tip mucilage which is stored in a periplasmic compartment at the conidial apex that can be released once conidia gets hydrated causing a spore wall rupturing (Hamer et al. 1988).Plant pathogenic fungi affect crops at various life cycle stages and plant tissues, including seeds, root and leaf development, and inflorescence (Gonzalez et al. 2011; Serrato-Diaz et al. 2014; He et al. 2019; Zhang et al. 2020). Moreover, to infect their hosts, fungi use different penetration routes; *M. oryzae* has shown to produce a dome-shaped appressorium cell that has a chitin and inner layer melanin enriched cell wall. The formation of a melanin layer is important to induce appressoria by generating a high osmotic pressure through glycerol accumulation (Howard et al. 1991). Other types of fungi have shown to penetrate their host through the stomata (Thomma et al. 2005).

Fungal plant pathogens are divided into three different groups based on their pathogenic lifestyle and the way they feed on the host (Doehlemann et al. 2017) : (1); biotrophic pathogens have evolved to match the life cycle of their host and their survival is dependent on their host, therefore they are known

to persist and utilize plant living tissues during their interactions (Doehlemann et al. 2017). Most known obligate biotrophic pathogens are those causing powdery mildew and rust diseases (Doehlemann et al. 2017). Besides, it has been shown that obligate biotrophic species are not able to utilize a wide range of substrates as energy sources, and therefore they depend on their host as an energy source provider (Wernegreen 2005). This might be due to a loss of genes involved in metabolic pathways, such as genes in the nitrogen and sulfur assimilation pathways (Kemen and Jones 2012). Nonobligate biotrophic plant pathogens are more diverse than obligate biotrophs such as the *Basidiomycota, Ustilaginales* and certain species of *Ascomycota and Claviceptacea* as well. Nonobligate species can survive without colonizing a plant host (Doehlemann et al. 2017). An example includes the ergot disease fungus *C. purpurea* that is known to be a biotrophic pathogen in planta, but it can be easily grown outside the host in axenic culture (Tudzynski and Scheffer 2004),(2); necrotrophic pathogens, which kill their hosts to extract necessary nutrients for their survival as a result the plant dies by necrosis. Like obligate biotrophic pathogens, necrotrophic pathogens use different effector molecules such as enzymes and toxins and mechanisms to evade the host immune system (Choquer et al. 2007).Compared to biotrophic pathogens, necrotrophic pathogens include a much wider range of species that belong to diverse genera. Based on the production of host-specific toxins (HST) that play an important role in inducing pathogenicity on the host, necrotrophic pathogens are divided into narrow-host-range and broad-host-range species (Mengiste 2012). *Botrytis cinerea* and *Sclerotinia sclerotiorum* are the best examples of broad-host-range, necrotrophic plant pathogens. *B. cinerea* causes the gray mold disease. This disease is widespread and results in serious economic damage worldwide. Usually, the fungus penetrates the plant at an early stage of crop development and remains quiescent until the plant physiology changes. As a result, apparently healthy crops once harvested might cause serious damage. *B. cinerea* infects the plant host by conidia, which penetrate the cuticle through appressoria. At the end of the infection cycle, the fungus develops sclerotia to be able to survive within dying host tissues (Doehlemann et al. 2017), (3); hemibiotrophic pathogens, start the infection as biotrophs and then switch to become necrotrophs during the last stages of infection. Hemibiotrophic pathogens have evolved highly successful infection strategies, and they are considered as one of the most aggressive plant pathogens (Doehlemann et al. 2017). A great example of hemibiotrophic fungus is the causal agent of rice blast disease *M. oryzae*.

The annual yield loss due to rice blast disease can be up to 30% of total rice production (Wilson and Talbot 2009). In addition *M. oryzae* can also infect other hosts including wheat and other small grains (Cruz et al. 2012). During the early stages of the infection, *M. oryzae* derives nutrition from living cells, then it switches into the necrotrophic stage to kill their host cells for nutrition, followed by producing asexual spores at the last stages of infections. To infect their host successfully, *M. oryzae* conidia develop melanized appressorium, to allow them to penetrate through the rice cell wall by inducing an enormous turgor pressure (Dagdas et al. 2012). Once it has successfully penetrated to the plant cell, the primary hyphae spread through plant cells and switch into bulbous invasive hyphae that are sealed inside the plant membrane structure (Kankanala et al. 2007). Subsequently, the primary hyphae of *M. oryzae* invade adjacent cells via plasmodesmata and switch to the necrotrophic stage, forming typical necrotic lesions on the surface of the rice leaves (Ebbole 2007).

Plants have developed two types of innate immune mechanisms: (1); pathogen-associated molecular patterns (PAMPs) triggered immunity (PTI); and (2) effector triggered immunity (ETI) playing an essential role on overcoming the invasion of pathogens (Suffert et al. 2011). PAMPs are usually fungal components. The best known fungal PAMP is chitin. Other characterized fungal PAMPs include endopolygalacturonases (Zhang et al. 2014). The gene coding rice chitin elicitor-binding protein (CEBiP), a cell surface-localized receptor, was the first chitin immune receptor gene to be cloned (Kaku et al. 2006). Such receptor plays an important role in chitin recognition and the induction of chitin-triggered immunity (Kaku et al. 2006). PAMPs and microbe associated molecular patterns (MAMPs) are detected by plant pattern recognition receptors (PRRs) (Boutrot and Zipfel 2017). Interactions between PRRs and PAMPs are essential to activate downstream signaling leading to the initiation of first reaction of defense called PAMP triggered immunity (PTI). PTI induces diverse immune responses including reactive oxygen species (ROS) burst, expression of immunity related genes and accumulation of antimicrobial compounds (Larroque et al. 2013). To facilitate infection, fungi must be able to overcome PTI defenses. Hence, the fungal pathogens have evolved mechanisms to evade the plant host immune system, such as physical and chemical barriers (Cook et al. 2015). To suppress the immune response and manipulate host cells, plant pathogens secrete virulence factors called effector proteins

(Stergiopoulos and de Wit 2009; Giraldo and Valent 2013). Effectors are molecules that modify host cell structure and function and are able to evade plant defenses to facilitate infection. Effectors are grouped into two classes based on their target sites in the host plant (Kamoun 2006). While cytoplasmic effectors are translocated within the plant cell, apoplastic effectors are able to interact with extracellular targets and surface receptors once secreted into the apoplast of the plant (Djamei et al. 2011; Park et al. 2012). Effectors are essential in suppressing PTI. Moreover, they are also recognized by the plant immune system which in turn triggers the second line of immune response called effector triggered immunity (ETI). During ETI response, effectors are recognized by plant resistance proteins (R proteins), which are intracellular nucleotide-binding leucine rich repeat (NLR) receptors. NLRs are able to recognize the effectors, either directly or indirectly (Qi and Innes 2013). The activation of ETI pathway by effectors cause disease resistance and as a result hypersensitive cell death response (HR) occurs at the infection site (Jones and Dangl 2006; Gao et al. 2017; Zavaliev et al. 2020). Plant R genes confer gene-for-gene resistance to a wide range of virulence genes by recognizing pathogen avirulence gene (Avr gene) products that initiate downstream signaling leading and mobilize a response to the invading pathogen. In turn, fungi must evolve new virulence factor allowing them to evade recognition and overcome immune defenses. So, plants and their pathogens have been found to be engaged in a constant arms race. Arms races occur at the level of genes encoding plant receptors, which detect and recognize pathogens and genes coding for effectors in pathogens enhancing infection by evading the plant defenses (Sánchez-Vallet et al. 2018). The arms race between plant host and pathogens can continuously impose selection pressures on each other. Such selection pressure led to the evolution of pathogen isolates that have either lost or modified the recognized effector or gained novel effectors to evade plant defenses. Moreover, the arms race between plant and pathogens resulted also in the evolution of novel plant receptors that either recognize the modified effectors or the recently acquired effectors (Houterman et al. 2008; Howlett et al. 2015; Seong et al. 2020).

Many fungal effectors and plant resistance genes have been identified. For example, for a successful infection, *M. oryzae* evades the host immune response by inducing oxidative burst or plant cell death during the biotrophic stage. Such suppression is achieved by secreted effector proteins such as *Slp1*,

that is known to accumulates at the interface of fungus-rice cells during the early stages of infection and competes with *CEBiP* (a chitin-binding protein of rice) for chitin oligosaccharides binding through *LysM* domains, thus induce chitin-triggered immunity in the host (Mentlak et al. 2012).The hemibiotrophic fungus *Leptosphaeria maculans* responsible for stem canker (Blackleg) of oilseed rape (*Brassica napus*).This disease is mainly controlled using specific *R* genes often combined with quantitative resistance (Hubbard et al. 2020). About 7 Avr genes from *L. maculans* have been cloned and most are located in repeat-rich, gene-poor genomic regions (Rouxel and Balesdent 2017).For example, the *AvrLm1* is recognized by two *plant R* genes, *Rlm1* and *LepR3*. These resistance genes are located on different chromosomes and are encoding different R proteins (Larkan et al. 2013). It has been shown that the virulence toward *Rlm1 is* achieved through a deletion mutation of *AvrLm1* and its surrounding region (Gout et al. 2007). Moreover, recently, *AvrLm1* was found to be recognized by the R protein LepR3 (Larkan et al. 2013). *AvrLm4-7* are recognized by *Rlm4* and *Rlm7* resistance genes. The virulence against *Rlm4* is achieved through a single non-synonymous mutation (Blondeau et al. 2015). Deletion mutations of effectors have also been reported to cause virulence against *Rlm7* (Blondeau et al. 2015). For the rice disease causing fungus *M. oryzae,* seven Avr genes have been cloned (Liu et al. 2013). Interestingly, most of them are involved in complex interactions, as a result two "cooperating" R genes are necessary to recognize each Avr gene (Okuyama et al. 2011; Cesari et al. 2013; Takagi et al. 2013). Several *Fol* Avr genes were identified in the soil fungus infecting tomato *Fusarium oxysporum f.*sp. *lycopersici* (*Fol*) such as *Avr1* (recognized by *R* genes *I* and *I-1*), *Avr2* (recognized by *I-2*) and *Avr3* (recognized by *I-3*) (Houterman et al. 2008). It has been demonstrated that *Avr3* and *Avr2* are important for full virulence of *Fol* on tomato. Moreover, no deletion mutation events, or *SNP*s were identified to prevent their recognition by *I-3* resistance genes, while three *SNPs* preventing recognition by *I-2* without modifying their virulence have been identified (Lievens et al. 2009). The wheat pathogen *Zymoseptoria* tritici produce a virulence phenotype through the *AvrStb6* gene that has been shown to encode small, cysteine-rich, secreted protein matching wheat cultivars carrying the *Stb6* resistance gene (Zhong et al. 2017). For *Cladosporium fulvum* (syn. *Passalora fulva*), a highly specialized plant pathogen infecting tomato, the first Avr gene to be identified in the *C. fulvum*–tomato interaction is the putative *Avr9* avirulence gene of *C. fulvum*, matching the *Cf-*

9 resistance gene on tomato. The *Avr9* gene codes a 28-amino-acid (aa) peptide (Schottens-Toma and de Wit 1988) and plays an important role in inducing the immune hypersensitivity response (HR) in the injected area of *Cf-9*-containing plants (Scholtens-Toma et al. 1989). The *Avr4* gene is another important virulence gene identified in the *C. fulvum* that matches the tomato *Cf-4* resistance gene. The *Avr4* gene encodes a 135-aa preproprotein with a characteristic signal peptide for extracellular targetin (Van Esse et al. 2007).

Adaptation mediates fungal pathogens genetic diversity

In their natural environment, fungi are consistently exposed to a variety of fluctuating conditions that might threaten their life and infectious potential including changing climatic conditions, wide range of fungicide and different host immune responses (Croll and McDonald 2017; Li, Lu, et al. 2020; Tibpromma et al. 2021). To meet and adapt to their hosts and environments, fungi have evolved a diversity of genetic and physiological traits. The diverse genetic traits of fungal population is a result of the interaction between different forces including mutation, sexual reproduction and mating system, gene flow, random drift, and selection (Tisserant et al. 2013; Çelik Oğuz and Karakaya 2021).Genetic diversity enables rapid increases in virulence and antifungal drug resistance. Mutations are a major source driving genetic variation (Bamba et al. 2019; Tsui et al. 2019). Mutations cause changes in the DNA sequence of genes, and these generate new alleles in populations. As a result, change in pathogen populations have seen over the years and new virulent or fungicide resistant genotypes of plant pathogens can be formed through mutations and these could break major resistance genes in plants or fungicide efficacy (Kolmer 1996; Mes et al. 2000). One example includes the banana pathogen *Fusarium oxysporum sp. Cubense (Foc)*, which has evolved diverse effector proteins called Secreted In Xylem (SIX) molecules that interact with the defense responses of the host plant. There are a large number of diverse *Foc* isolates called races in which pathogenic variation exists in relation to different cultivars of the same host species (Czislowski et al. 2018). Moreover, *F. oxysporum f. sp. lycopersici (Fol)*, a tomato fungal pathogen, some isolates of this fungus secrete an effector that can both trigger and suppress R gene-based immunity. This effector is called *Avr1* and causes disease resistance when

pathogens interfere with the R gene (I or I-1) carried by the tomato plant (Houterman et al. 2008). Horizontal genes transfer (HGT), which is defined as the stable integration of genetic material due to the transfer between individuals, is considered another key mechanism to create novel virulent genotypes. In fungi, there are several studies on the impact of HGT on potential emergence of fungal plant pathogens including the HGT of the *ToxA* gene from *Stagonospora nodorum* to *Pyrenophora tritici-repentis* enabled the latter fungus to increase wheat disease severity (Mehrabi et al. 2011). Furthermore, because fungicides are applied on a regular basis throughout the year, this might lead to the emergence of newly evolved pathogens. Plant pathogen populations are typically characterized by a low frequency of resistant individuals who do not impede disease control in the open field. Disease control issues can arise when resistant individuals outnumber sensitive individuals. The interaction of various factors, such as the fungicide's mode of action and utilization, pathogen biology, and epidemiology, determines the evolution of fungicide resistance in a population (Massi et al. 2021). Fungicide resistance can be conferred by different mechanisms including: (i) changes in the target site that alter fungicide binding; (ii) overproduction of the target protein; (iii) an active efflux reducing the uptake of fungicide (Acero et al. 2011). Over years, fungi have adapted quickly to changing environments including overcoming fungicide-based crop protection strategies. This is usually achieved by single site mutations in the gene encoding for the fungicide and could cause decreased sensitivity (Zhang, Imran, et al. 2020). Although multisite fungicides are believed to associate with a lower risk of resistance evolution as this requires that multiple mutations in different fungicide resistance genes happen simultaneously to cause multidrug fungicide resistance. Multifungicide drug resistance has developed in some plant pathogenic fungi in the field (Omrane et al. 2017).

Sexual recombination also provides a powerful mechanism of genetic variation (Charlesworth 2009; Stefansson et al. 2014). Fungi have complex life cycles that include different modes of reproduction. Almost all fungi can reproduce asexually (clonally) through the formation of mitotic spores or reproduce sexually through meiotic recombination to generate meiotic spores (Nieuwenhuis and James 2016). During the sexual reproduction, recombination creates many new allele combinations, producing many genotypes that might increase the pathogen's adaptability in new environments. Hence, restricting fungi to asexual reproduction has seen to play an important role in the management of crop diseases.

Introduction of sexual reproduction in the life cycle of the *Z.tritici*, which causes Septoria tritici blotch on wheat has been recognized as an important factor in the disease management (McDonald and Mundt 2016). The management included the induction of small population size through reducing the fungal sexual reproduction by removing the sexual stage of *Z. tritici* by tillage (McDonald and Mundt 2016). *Plasmopara viticola*, the causal agent of grapevine downy mildew, reproduce either sexually or asexually. Their high reproductive ability had the major advantage of enabling them to quickly evolve to adapt to changing environments. (Calo et al. 2013). Moreover, mutations that might happen after each reproductive cycle can also provide fungicide resistance advantage (Hawkins and Fraaije 2018). Although, most evolutionary forces tend to increase the genetic diversity of fungi to allow them to adapt to different environments, evolutionary forces such as genetic drift defined as a process in which allele frequencies within a population change by chance might reduce the total amount of genetic variation within a population (Latorre et al. 2020).

Transposable elements: source of genetic diversity and drivers of adaptive evolution in pathogens

Transposable elements (TEs) are a class of repetitive mobile genetic elements that were first discovered in maize (McClintock 1950). In eukaryotes, the repeat content can differ between species, they occupy almost half, 46%, of the human genome (Lander et al. 2001), up to 85% in maize (Vicient 2010), whereas fungal plant-pathogen genomes covers a wide range of TE content that can cover up to 90% of the genome content as in powdery mildew pathogenic fungi *Blumeria graminis* (Frantzeskakis et al. 2018). Depending on species, TEs can be located in different regions of the chromosome such as genic, pericentric or centromeric regions (Mat Razali et al. 2019). There are two major categories of TEs based on their replication mechanism, namely class I TEs, that transpose using an RNA intermediate (i.e., RNA transposons) and class II TEs, that transpose using a cut and-paste mechanism (i.e., DNA transposons) (Wicker et al. 2007). Additionally, the TE classes can be divided into a variety of families and superfamilies based on their coding sequence structure (Wicker et al. 2007). Some TEs (e.g., LTRs) are autonomous because they encode the necessary machinery to execute their own transposition.

Several categories of TEs lack their own coding ability and are called non-autonomous TEs (e.g., MITEs) relying on proteins encoded by other ('autonomous') TEs (Wicker et al. 2007).

Fungal pathogen genomes are plastic and harbor often many insertions of TEs. These TEs are considered as the major driving force for adaptive genome evolution. Such TE-based adaptive evolution favors the generation of genetic variation within fungal populations and allowing fungi to adapt to changing environments, favoring their capacity in evading plant immune system, as well as overcoming fungicide-based crop protection strategies. TEs play a role in adaptive genetic variation through: (1) TE insertion into coding genes, however, most TEs have seen to avoid insertion into coding sequences (Jangam et al. 2017; Curcio 2019), (2) TE insertion into introns or (3) TE transposition in proximity to genes (Casola and Betrán 2017). Moreover, TEs insertion in the genome are subject to natural selection where deleterious insertion that affects host fitness will be eliminated, while advantageous and neutral insertions will be maintained in the genome (Baucom et al. 2009; Stritt et al. 2018a). Evolving fungi carry important virulence, fungicide resistance or stress adaptation associated genes that have been shown to be located in TE-rich regions (Grandaubert et al. 2014; Soyer et al. 2015; Omrane et al. 2017; Torres et al. 2020). During infections, fungi use molecules called secondary metabolites to manipulate their plant hosts (Brakhage 2013) such as Melanin, a well-known secondary metabolite used by many fungi during host colonization and survival under stress conditions (Krishnan et al. 2018). An insertion of TEs upstream of the *Zmr1* promoter *Zmr1,* which controls expression of the genes in the melanin biosynthetic gene cluster, was contributing to variation in melanin accumulation and playing important role in fungal survival in stressful environments (Krishnan et al. 2018). Similarly, TE insertions have been shown to contribute to the pathogenicity increase in *F. oxysporum*, a Fusarium wilt being one of the most important and destructive diseases in banana crops worldwide (Ma et al. 2010). TEs have an important impact on the potential to adapt to new environment and overcome fungicide-based crop protection strategies. Multi-drug fungicide resistance was achieved through TE insertions (Omrane et al. 2017). For example, the wheat pathogen *Z. tritici*, for which disease control relies mainly on resistant wheat cultivars and fungicide applications. The fungus has a high potential to overcome both control methods. Resistance against all used fungicides has been observed over decades. Insertions of TEs in

the *MFS1* promoter controlling the *MFS1* transporter gene was found to induce this gene overexpression and to enhance fungicide resistance in *Z. tritici* (Omrane et al. 2017). In plant pathogenic fungi, TEs can act as a potent agent mediating the genetic variation and driving phenotypic evolution, yet many TEs are difficult to identify due to their complex nature. The recent advances in TEs identification methods might accelerate the identification of such TEs and mediate adaptive evolution in plant pathogens.

Evolving plant pathogens require monitoring

Pathogenic fungi are known to cause major food insecurity and economic losses (Horbach et al. 2011; Avery et al. 2019). Crop protection is primarily achieved through the application of a variety of fungicides and resistance breeding (Nelson et al. 2018; Corkley et al. 2022). However, fungal pathogens have evolved resistance to major fungicides currently in use (Mohd-Assaad et al. 2016; Massi et al. 2021; de Chaves et al. 2022). In addition, efforts to breed resistant crop varieties have repeatedly been defeated by rapid evolutionary change in pathogen populations allowing to circumvent resistance mechanisms (Nelson et al. 2018). Fungal plant pathogen populations that evolved resistance to specific fungicides harbor numerous point mutations in or nearby the genes encoding the targets of the chemical compounds (Deising et al. 2008; Bowyer et al. 2012; Mohd-Assaad et al. 2016; Omrane et al. 2017). Similarly, pathogen populations virulent on previously resistant crop varieties have often mutated or deleted a specific set of genes that encode proteins recognized by the plant immune system (Kashiwa et al. 2016; Hartmann et al. 2017; Hartmann 2022; Olivera et al. 2022). Monitoring the evolution of more virulent or fungicide resistant plant pathogens strains would be important for crop disease management.

Monitoring of fungicide resistance or virulence mutations is mainly achieved through the sequencing of target genes. Nowadays, fungicide resistance is particularly problematic for the triazole or demethylase inhibitor (DMI) class of fungicides (Parker et al. 2014, p. 51) which play an important role in control diseases of plants (Poole and Arnaudin 2014). Therefore, the management of fungicide

resistance has become a threat, and many methods have been developed that can be used to detect the evolution of a resistant population of a pathogen including in vitro fungicide sensitivity assays (Saville et al. 2015; Samils et al. 2021). Such analyses require the isolation and culturing of individual fungal strains that can then be tested for growth on media containing different fungicide concentrations. The fungicide dose that effectively inhibits growth by 50% is determined for comparison among samples (*i.e.*, EC50) (Saville et al. 2015; Wang et al. 2017). Although there is some controversy over the practicality of this method, this one is laborious and limited to fungal species that can be cultured in absence of the host.

With advances in molecular techniques and the characterization of mutations associated with fungicide resistance, a number of genetic screening methods have been developed. An ideal mutation screening system should be sensitive, not time consuming and cost effective. These methods includes Sanger sequencing, TaqMan assays based on fluorescently-tagged, allele-specific probes (Schleinitz et al. 2011) and can be used to detect single nucleotide polymorphisms (SNPs), however, such screening approaches are labor-intensive and have low potential for multiplexing large numbers of individual loci. Virulence surveillance of fungal plant pathogens has been implemented for the wheat rust pathogen *Puccinia graminis f. sp. tritici* based on simple sequence repeat (SSR) markers (Zhong et al. 2009; Visser et al. 2011) and used to differentiate the virulent Ug99 race lineages (Jin et al. 2008; Visser et al. 2011). However, these SSR makers have been less useful in distinguishing differentiation within the Ug99 race (Singh et al. 2015). The tomato wilt pathogen called *F.oxysporum f. sp. lycopersici* (*Fol*) was monitored using loop-mediated isothermal amplification (LAMP) to distinguish the pathogenic race 3 from other races based on point mutations in the secreted in xylem 3 (SIX3) gene characteristic of race 3. Moreover, (LAMP) assays have also been applied to detect fungicide resistance in *Fusarium graminearum* (Duan et al. 2014) and *Sclerotinia sclerotiorum* (Duan et al. 2016). However, LAMP assays is considered to be expensive due to the high costs of individual probes (Ayukawa et al. 2017). More recently, NGS based techniques have been developed including whole genome sequencing (WGS) that can be used to detect single nucleotide polymorphisms (SNPs) and structural variation (Cao et al. 2016). Applications of WGS have contributed to the mapping and characterization of virulence

and resistance factors primarily through genome-wide association mapping (Mohd-Assaad et al. 2016; Zhong et al. 2017; Pereira et al. 2020; Hartmann 2022). Additionally, restriction-site-associated DNA sequencing (RAD-seq) and Genotyping-by-Sequencing (GBS) were used, both methods rely on restriction enzymes to reduce genome size and complexity and exploring SNPs adjacent to restriction enzyme sites (Baird et al. 2008; Elshire et al. 2011). However, such genotyping approaches assess only mutations near restriction enzyme cut sites. More recently, PacBio long read assay have been developed to screen fungicide resistance mutations in *Z.tritici* populations (Samils et al. 2021) but due to the varying amplicon sizes generated by this assay two separate multiplex PCRs are required to separate shorter and longer amplicons (Samils et al. 2021).

Plants are often co-infected by diverse pathogens

Recent advances in metagenomics have highlighted the vast diversity of the plant pathogen community (Schenk et al. 2012). Plants are simultaneously colonized by multiple pathogens in theirs environment (Brown et al. 2010; Anfoka et al. 2016; Xu et al. 2022). Colonizing microorganisms often occur as complex communities with complex interkingdom (mainly bacteria and fungi) interactions on single plant host (Salvaudon et al. 2013; Susi et al. 2015; Tollenaere et al. 2017; Ontiveros et al. 2022). Plants can be colonized by multiple pathogens, not only of different kingdoms but also different species or strains (i.e. genotypes) of the same species (Hortal et al. 2016). Colonizing by multiple pathogenic organisms are knowns as coinfection and are very common in nature (Susi et al. 2015). As a result, a successful plant defense system will use several resistance (R) genes that work together to trigger an effective response to multiple attacks. Plant genomes encode a huge number of R-genes that recognize plant pathogens and trigger an affective immune response (Dangl and Jones 2001). Co-infection might have different impacts on disease severity depending on the outcome of microbial interactions and host immune responses (Abdullah et al. 2017). Although, previous studies showed that plants co-infected by different soil-borne pathogens showed a significant increase in disease severity compared with single infection (Pfender and Hagedorn 1982). Some other have been shown that co-infection by different soil-borne pathogens do not necessarily lead to more severe disease than single infection (Vandemark

et al. 2010). Co-infection outcomes can either lead to cooperation or competition depending on the co-infecting strains (Vandemark et al. 2010). Additionally, such outcomes can be affected by the order of co-infection, plant immune response as well as the genotypes of co-infecting strains (Aghnoum and Niks 2012; Marchetto and Power 2018; Barrett et al. 2021).

Intraspecific co-infections are playing a key role in plant disease progression with fungal plant pathosystem receiving most attention (Schürch and Roy 2004; Susi et al. 2015; Barrett et al. 2021). Within fungal pathogen species, strains can show a high degree of variation in virulence (Schürch and Roy 2004; Barrett et al. 2021). Intraspecific competitive interactions between fungal isolates can have impact on fungi and plants and be influenced by several environmental abiotic factors (Hortal et al. 2016). The virulence of co-infecting *Podosphaera plantaginis* strains can be increased on the *Plantago lanceolata* host (Susi et al. 2015). In other systems such as *Z.tritici* infecting wheat, virulence appears unaffected by co-infections (Schürch and Roy 2004; Barrett et al. 2021). However the trajectory of an infection depends on the host genotype and genotypes of competing strains (Barrett et al. 2021; Bernasconi et al. 2022). Besides, Bernasconi et al. (Bernasconi et al. 2022) showed that the competitive advantage of individual strains genotypes for transmission is not associated with virulence among the strains (Bernasconi et al. 2022). The growth of mixed genotypes outside of the host is only poorly associated with the success of the same genotype mix on the plant though (Bernasconi et al. 2022). The complex links between competitive abilities of the pathogen and consequences for the plant host raise important questions how co-infections impact evolutionary trajectories of host-pathogen systems.

The fungal wheat pathogen *Zymoseptoria tritici*

The fungus *Z.tritici* is a haploid, heterothallic ascomycete that causes one of the economically most important wheat diseases called Septoria tritici blotch (Goodwin et al. 2011; Fones and Gurr 2015). The pathogen has emerged at the onset of wheat domestication in the Middle East (Stukenbrock et al. 2007) and has since spread to all wheat-producing areas of the world (Zhan et al. 2003). The genome of *Z. tritici* contains 13 core chromosomes and up to 8 accessory chromosomes, which are not present in all

strains of the species. *Z. tritici* is considered as a dimorphic fungus, depending on culture conditions it can grow either as the yeast-like form called blastospores or as filamentous hyphae. Hyphae might be formed from either germinated sexual spores or pycnidiospores (asexual spores) and they play an important role in wheat leaves penetration through stomata during infection. The environmental conditions can affect the morphology of *Z. tritici*. Nutrient-rich media and temperatures ranging from 15 °C to 18 °C has shown to induce blastosporulation whereas nutrient-limited media and high temperatures (from 25 °C to 28 °C) lead to the transition of the blastospore-to-hyphae in the *Z. tritici* (Francisco et al. 2019). Moreover, it has been demonstrated that *Z.tritici* are able to survive better high cold or heat based stressful conditions through the formation of a new morphotype called chlamydospores (Francisco et al. 2019). Only few genes involved in *Z. tritici* dimorphism have been identified and most of them are belonging to the mitogen-activated protein kinase *MAPK* and *cAMP-PKA* signaling pathways. The *ZtHOG1* gene, encoding a well conserved mitogen-activated protein kinase *ZtHog1p*, that has been shown to be essential in the osmotic stress response in many fungi (Mehrabi et al. 2006). Besides, the inactivation of the cyclin-encoding gene *ZtMCC* has shown to delay filamentous growth, as well as increasing melanin biosynthesis (Choi and Goodwin 2011). Another gene identified is the *ZtALG2* gene that is involved in secreted proteins glycosylation. The mutant lacking the *ZtALG2* gene was unable to infect wheat as well as in switching from yeast to filamentous growth (Marshall et al. 2011). *Z. tritici* has a mixed reproduction system involving sexual and asexual reproduction. Several sexual cycles in *Z.tritici* has shown to occur during the growing season (Kema, Yu, et al. 1996; Hunter et al. 1999; Duvivier et al. 2013), they require a physical encounter between two compatible strains (Mat1-1 and Mat1-2) (Kema, Verstappen, et al. 1996), and generally occurs on senescent tissues, with pseudothecia development 46 to 76 days after the initial infection in field (Suffert et al. 2011). It should be considered that the vast majority of crossing events occur when leaf lesions caused by two compatible strains either coalesce or are located very close together (Orellana-Torrejon et al. 2022). Such sexual reproduction plays an essential role in increasing and maintaining the diversity of local pathogen populations as well as allowing them to adapt to different selection pressures, such as those exerted by resistant hosts or fungicides use. The genetic diversity of *Z. tritici* has been well studied over the years. It has been shown that this fungus has a high levels of genetic diversity and rates of

population structure and gene flow that vary depending on the sampled geographical area and/or the marker system used on bread wheat (El Chartouni et al. 2011; Gurung et al. 2011).The high diversity of *Z. tritici* either in field or wheat leaves might result on constant mating in the populations, moreover, the ascospores could provide primary as well as secondary inoculum (Morais et al. 2019). Besides, it has been shown that avirulent strains can be maintained asymptomatically "on" or "in" leaf tissues of wheat carrying the matching resistant gene for long enough and then reproduce sexually (Orellana-Torrejon et al. 2022). The infection cycle of *Z. tritici* can be divided into different phases: (1) entry of the fungus into the host, (2) colonization of the plant tissue and (3) formation of fruiting bodies (Steinberg 2015). Infection begins following the inoculation of pycnidiospores on the wheat leaf by rain splashes, the ascospores then germinate to switch into hyphae (Hilu and Bevee 1957; Duncan and Howard 2000). The fungal hyphae enter the leaf tissue through the stomata to colonize the substomatal cavity, which lead to hyphal colonization of surrounding tissue. This phase is biotrophic and *Z. tritici* is able to overcome plant immune response by suppressing or triggering very weak plant immune response causing an asymptomatic stage (Keon et al. 2007; Rudd et al. 2015). This phase is also characterized by a very slow hyphal growth in the apoplastic space and usually last for about 7-14 days (Kettles and Kanyuka 2016). Moreover, it has been shown fungus uses its own lipids and fatty acids as the main energy sources during this asymptomatic phase. The asymptomatic phase is also characterized by up regulation of the genes encoding secreted cutinases and lipases. Moreover, the fungus might also use host during this asymptomatic stage (Rudd et al. 2015). Pre-pycnidia are then formed in the colonized substomatal cavities (pre-pycnidia formation) and during this stage cells undergo programmed cell death. Fungi profit from the released dying plant nutrients to proliferate. This phase is referred to as necrotrophic symptomatic phase. This phase of infection phase is characterized by reprogramming of both host and pathogen transcriptomes, a strong activation of host defense responses and release of nutrients into the leaf apoplast (Keon et al. 2007; Rudd et al. 2015). Finally, pycnidiospores are generated from the matured pre-pycnidia. Pycnidiospores are then released through stomatal openings and can then be spread over millions of kilometers through water splash or wind to establish new infections sites (Steinberg 2015; Sánchez-Vallet et al. 2015; Kettles and Kanyuka 2016).

Chitin is an important structural component of fungal cell walls and acts as a pathogen-associated-molecular-patterns (PAMP) (Lenardon et al. 2010) that upon recognition by the R genes on plant host, triggers immune responses. To successfully establish an infection. *Z. tritici* has evolved various strategies to overcome chitin-triggered plant immunity, such as the secretion of effector proteins to prevent the activation of chitin-induced immunity. LysM effector proteins family are one of *Z. tritici* secreted effector (Tian et al. 2021). Previously these effectors were shown to protect fungal hyphae against host chitinases and suppress chitin-induced reactive oxygen species (ROS) (Marshall et al. 2011; Tian et al. 2021). Plant host have evolved resistance genes (R) to recognize effector genes (McDowell and Woffenden 2003). For *Z. tritici* to remain successful in infecting the host, the pathogen needs to evolve virulence towards novel R gene products carried by newly selected cultivars. An example includes the small, cysteine-rich, secreted protein called *AvrStb6* which produces an avirulence phenotype on wheat cultivars carrying the *Stb6* resistance gene (Zhong et al. 2017). *Avr3D1* is another known rapidly evolving small, secreted protein that is able to escape from recognition by resistance proteins *Stb7* on wheat (Meile et al. 2018).

Over time, crop protection including Septoria tritici blotch against plant pathogens relies on the use of different fungicides (Corkley et al. 2022). However, repeated use of fungicides led to the evolution of resistance within *Z.tritici* strains (Hartmann 2022). Mutation is the engine of evolution as it generates the genetic variation on which the evolutionary process depends. Major routes to resistances included the rise of mutations in genes encoding the targets of the fungicide, in particular in *CYP51* encoding the target of azoles (Mohd-Assaad et al. 2016; Corkley et al. 2022). The rise of succinate dehydrogenase inhibitor (SDHI) resistance mutations are the most recent of the observed gains in resistance (Fungicide Resistance Action Committee, FRAC, 2021). Association mapping in *Z. tritici* has recently revealed specific mutations underlying the gain of virulence on previously resistant wheat cultivars including cultivars carrying the resistance gene *Stb6* and others (Zhong et al. 2017; Meile et al. 2018; Singh, Karisto, et al. 2021). Hence, as evolving *Z. tritici* strains pose a real risk to global food production a continuous monitoring of the strain evolution is crucial. Monitoring of fungicide resistance mutations is mainly achieved through the sequencing of target genes including the recent development of long-

read sequencing assays (Samils et al. 2021). A joint monitoring of virulence mutations and genetic diversity is lacking though.

Furthermore, the completion of several genome sequencing projects and advances in TEs insertion detection methods clarified the role of TEs in genome evolution. Studies have shown that TE-mediated mutations play an important role in the adaptive evolution. Such repeated sequences vary in content among genomes (Badet et al. 2020). In addition, accessory chromosomes appear to contain higher proportions of TEs than the core chromosomes (Croll and McDonald 2012; Grandaubert et al. 2015). It is thought that TEs in *Z. tritici* are involved in pathogenicity through several mechanisms (i); TE insertions based effector genes diversity (Krishnan et al. 2018; Meile et al. 2018); (ii) causing gene gain or loss or; (iii) leading to large scale chromosomal rearrangements (Croll et al. 2013; Plissonneau et al. 2016). *Avr3D1* has been shown to reside in a cluster of putative effector genes located in a genome region rich in TEs insertions (Meile et al. 2018). Similarly, numerous TEs have been identified in *Z.tritici* that affect expression of genes related to fungicide resistance. Three TEs insertions in the *MFS1* promotor disrupt the gene expression of *MFS1* gene coding *MFS1* transporter causing efflux overexpression (Omrane et al. 2017) and thereby promoting the emergence of novel resistant pathogen strains. TEs are important for generating variability and increased fitness in new or challenging environments. Such stresses may be abiotic (*e.g.* temperature) or biotic, such as pathogen infections. TE activity under stressful conditions has been found to de-repress the transposition activity of TEs during infection (Fouché et al. 2019). Through studying the TE structure in the genome, it has been found that such insertions are associated with genome expansion. The TEs might accumulate also in populations outside of the center of origin (Oggenfuss et al. 2021). This suggests that relaxed purifying selection plays an important role in maintaining TEs in such populations. We still lack knowledge on the evolutionary dynamics of TEs associated with both virulence and fungicide resistance evolution.

Genetic diversity driven evolution facilitated *Z. tritici* to diversify phenotypically (Linde et al. 2002). In the field, different strains can co-infect the same wheat host (Linde et al. 2002). Co-infection can have effects on disease severity, transmission, and pathogen virulence. Besides, previous studies

showed that mixed infections can reduce the production of reproductive structures called pycnidia (Halperin et al. 1996; Schürch and Roy 2004). Moreover, co-infection altered transmission potential of *Z.tritici* fungal plant pathogen (Barrett et al. 2021). The concept of competition can be defined as the interaction occurring when organisms share a common, limited resource leading to a change in their fitness (Lang and Benbow 2013). Earlier work showed that by measuring the growth of different strains of *Z.tritici* in single and mixed cultures under nutrients deprived conditions that strains tend to compete for the limited sources and the slow growing strain alone can increase its growth rate during mixed culture and therefore benefit from the presence of co-inoculated strain (Bernasconi et al. 2022). However, the competitive outcome of such mixed interactions inside and within plant host by using a large number of conspecific genotypes is poorly studied.

Main objective of this thesis

Research on *Z. tritici* has made a lot of progress in recent years. But most of this research had little interest in developing monitoring tools to detect evolution of pathogen populations to allow a sustainable management of Septoria tritici blotch. Besides, despite the fact that *Z. tritici* forms genetically diverse infections in nature, consequences of co-infections are often poorly understood. Moreover, TEs play an important role in shaping phenotypic traits of adaptive significance but little is known about within species TEs insertions dynamics in lobal populations. In this thesis, I worked on improving detection of newly evolved genotypes of *Z. tritici* as well as studying TEs global distribution driving its adaptive evolution. I also highlighted the outcomes of mixed interactions inside and outside of the plant host and their impact on disease severity.

Chapter 1: A highly multiplexed assay to monitor virulence, fungicide resistance and gene flow in the fungal wheat pathogen Zymoseptoria tritici

Plant fungi evolve constantly to circumvent fungicide control as well as to overcome plant immune responses 2/22/23 1:52:00 PM(Houterman et al. 2008; Zhong et al. 2017; Yang et al. 2019; Pereira et al. 2020), Gains in virulence or fungicide resistance are often associated to mutations events. However, to reduce damage caused by fungal plant pathogens, a less time consuming and accurate detection of mutations associated with virulence and fungicide resistance evolution is crucial. So, I developed a new genotyping assay allowing a joint detection of virulence, fungicide resistance as well as assessment of genetic diversity in fungal plant pathogen field populations.

Chapter 2: Divergent outcomes of direct conspecific pathogen strain competition and during plant co-infection with consequences for disease dynamics

Pairwise co-infections are common in natural plant populations (Fang et al. 2021; Ontiveros et al. 2022; Susi et al. 2022). They can have important effects on disease severity and virulence evolution (Alizon et al. 2013; Tollenaere et al. 2016; Abdullah et al. 2017). Despite the importance of co-infections, providing a comprehensive picture of their consequences through investigating their outcomes by using

a large number of tested pairs are still challenging. In this chapter, I performed systematic pairwise interaction assays within and outside the plant host to investigate the possible outcomes of such interactions. I further investigated their consequences on disease dynamics.

Chapter 3: Diverse sets of transposable elements contribute to the rapid sequence evolution of virulence and fungicide resistance loci in the fungal wheat pathogen Zymoseptoria tritici

Transposable elements (TEs) are a class of repetitive mobile genetic elements (McClintock 1950). TEs can have an impact on virulence and fungicide resistance evolution (Omrane et al. 2017; Porquier et al. 2021). Therefore, they play an important role in mediating adaptive phenotypic variation significant for adaptation to changing environments. However, most studies are focused on within species individual insertions, whereas investigations of global population TE dynamics are largely non-existant. In this chapter, I used genome assembly datasets to investigate within species populations large scale mapping of TEs dynamics in fungicide resistance and virulence associated loci.

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Chapter 1

A highly multiplexed assay to monitor virulence, fungicide resistance and gene flow in the fungal wheat pathogen *Zymoseptoria tritici*

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Abstract

Crop pathogens pose severe risks to global food production due to the rapid rise of resistance to pesticides and host resistance breakdowns. Predicting future risks requires monitoring tools to identify changes in the genetic composition of pathogen populations. Here we report the design of a microfluidics-based amplicon sequencing assay to multiplex 798 loci targeting virulence and fungicide resistance genes, and randomly selected genome-wide markers for the fungal pathogen *Zymoseptoria tritici*. We optimized the primer design by integrating polymorphism data from 632 genomes of the same species. To test the performance of the assay, we genotyped 192 samples, including replicates, of *Z. tritici*. The fungus causes one of the most devastating diseases on wheat showing rapid adaptation to fungicides and host resistance. Analysis of the short-read sequence data generated by the assay showed a fairly stable success rate across samples to amplify a large number of loci. The performance was consistent between samples originating from pure genomic DNA as well as material extracted directly from infected wheat leaves. In samples with mixed genotypes, we found that the assay recovers variations in allele frequencies. We explored also the potential of the amplicon assay to recover transposable element insertion polymorphism relevant for fungicide resistance. As a proof-of-concept, we show that the assay recovers the pathogen population structure across French wheat fields. Genomic monitoring of crop pathogens contributes to more sustainable crop protection and yields.

Introduction

Approximately 30 percent of all crop diseases are caused by fungi (Jain et al. 2019). Plant pathogenic fungi affect crops at various life cycle stages and plant tissues, including seeds, root and leaf development, and inflorescence (Gonzalez et al. 2011; Serrato-Diaz et al. 2014; He et al. 2019; Zhang, Wang, et al. 2020). Yield reductions by pathogenic fungi cause food insecurity and economic losses (Horbach et al. 2011; Avery et al. 2019). Crop protection is primarily achieved through the application of a variety of fungicides and resistance breeding (Nelson et al. 2018; Corkley et al. 2022). However, fungal pathogens have evolved resistance to all major fungicides currently in use (Mohd-Assaad et al. 2016). In addition, efforts to breed resistant crop varieties have repeatedly been defeated by rapid evolutionary change in pathogen populations allowing to circumvent resistance mechanisms (Nelson et al. 2018). Predicting future breakdowns in fungicide efficacy and crop resistance remains challenging. Fungicide resistance is monitored across the European continent by analyzing mutations in known target genes related to the fungicide mode of action (Brent and Hollomon 1998; Klappach et al. 2020). However, the rise of pathogen strains defeating crop resistance is not comprehensively monitored. Notable exceptions include the screening of rust fungi (Park et al. 2011; Olivera Firpo et al. 2017; Cook et al. 2021; Fontyn et al. 2022). To reduce damage caused by plant pathogens, a timely and accurate detection of both fungicide resistance mutations and mutations associated with the defeat of crop resistance is essential.

Fungal plant pathogen populations that evolved resistance to specific fungicides harbor numerous mutations in or nearby the genes encoding the targets of the chemical compounds (Deising et al. 2008; Bowyer et al. 2012; Mohd-Assaad et al. 2016; Omrane et al. 2017). Similarly, pathogen populations virulent on previously resistant crop varieties have often mutated or deleted a specific set {Citation}of genes that encode proteins recognized by the plant immune system (Kashiwa et al. 2016; Hartmann et al. 2017; Hartmann 2022; Olivera et al. 2022). Fungicide resistance has traditionally been detected using in vitro fungicide sensitivity assays (Saville et al. 2015; Samils et al. 2021). Such analyses require the isolation and culturing of individual fungal strains that can then be tested for growth on media

containing different fungicide concentrations. The fungicide dose that effectively inhibits growth by 50% is determined for comparison among samples (*i.e.*, EC50) (Saville et al. 2015; Wang et al. 2017). The method is laborious and limited to fungal species that can be cultured in absence of the host. With advances in molecular techniques, a number of genetic screening methods have been developed including Sanger sequencing, TaqMan assays based on fluorescently-tagged, allele-specific probes (Schleinitz et al. 2011). In general, such screening approaches are labor-intensive and have low potential for multiplexing large numbers of individual loci. Virulence surveillance of fungal plant pathogens has been implemented for the wheat rust pathogen *Puccinia graminis f. sp. tritici* based on simple sequence repeat (SSR) markers (Zhong et al. 2009; Visser et al. 2011) and used to differentiate the virulent Ug99 race lineages (Jin et al. 2008; Visser et al. 2011). However, these SSR makers have been less useful in distinguishing differentiation within the Ug99 race (Singh et al. 2015). The tomato wilt pathogen *Fusarium oxysporum f. sp. lycopersici* (*Fol*) was monitored using loop-mediated isothermal amplification (LAMP) to distinguish the pathogenic race 3 from other races based on point mutations in the secreted in xylem 3 (SIX3) gene characteristic of race 3. However, LAMP assays can be expensive given costs of individual probes (Ayukawa et al. 2017). Therefore, there is a need for cost effective, sensitive, and quantitative genotyping tools that are able to identify specific mutations associated with pathogen emergence to improve early detection and effective disease management.

The advent of next generation sequencing (NGS) approaches has removed a series of limitations in pathogen monitoring. The most general application of NGS techniques is whole genome sequencing (WGS) that can be used to detect single nucleotide polymorphisms (SNPs) and structural variation (Cao et al. 2016).

Applications of WGS have contributed to the mapping and characterization of virulence and resistance factors primarily through genome-wide association mapping (Mohd-Assaad et al. 2016; Zhong et al. 2017; Pereira et al. 2020; Hartmann 2022). Low-cost, high-throughput methods based on NGS include reduced representation sequencing genotyping methods such as restriction-site-associated DNA sequencing (RAD-seq) and Genotyping-by-Sequencing (GBS), both methods rely on restriction enzymes to reduce genome size and complexity and exploring SNPs adjacent to restriction enzyme sites

(Baird et al. 2008; Elshire et al. 2011). However, such genotyping approaches assess only mutations near restriction enzyme cut sites. Applications in fungal pathogens mainly include assessments of recombination rates and mapping of quantitative traits in experimental crosses (Croll et al. 2015; Talas and McDonald 2015; Lendenmann et al. 2016; Stewart et al. 2018; Wyka et al. 2022). The analysis of individual regions involved in fungicide resistance has been improved by the recent development of a PacBio long-read sequencing assay based on the multiplex amplification of target genes in fungal wheat pathogen *Zymoseptoria tritici.* The main advantage is the ability to generate long-reads capturing significant haplotype information of individual strains revealing a series of alterations conferring increased resistance in response to different commercial fungicides. However, due to the varying amplicon sizes generated by this assay two separate multiplex PCRs were required to separate shorter and longer amplicons (Samils et al. 2021). High degrees of multiplexing for amplicons and samples were recently achieved using two parallel approaches for animal and plant species. Genotyping-in-thousands by sequencing (GT-seq) is based on multiplex PCR targeted amplicon sequencing to simultaneously genotype thousands of loci and hundreds of samples in a single Illumina sequencing run (Campbell et al., 2015). A limitation of this approach is the extended time required for its development (about ~4 months according to (Meek and Larson 2019). One challenge to overcome is imbalanced amplification of individual loci and samples. Such bias can be reduced by the use of microfluidics assays that physically separate sets of amplicons and samples (Kessler et al. 2022).

The fungal pathogen *Z. tritici* causes one of the economically most important wheat diseases called Septoria tritici blotch (STB) (Fones and Gurr 2015). The pathogen has emerged at the onset of wheat domestication in the Middle East (Stukenbrock et al. 2007) and has since spread to all wheat-producing areas of the world (Zhan et al. 2003). Populations have evolved resistance to all commercially used fungicides and repeatedly across continents (Hartmann 2022). Major routes to resistances included the rise of mutations in genes encoding the targets of the fungicide, in particular in *CYP51* encoding the target of azoles (Mohd-Assaad et al. 2016; Corkley et al. 2022). Furthermore, upregulation of the transporter gene *MFS1* due to the insertion of transposable elements in the promoter region contributed to azole resistance (Omrane et al. 2017). The rise of succinate dehydrogenase inhibitor (SDHI)

resistance mutations are the most recent of the observed gains in resistance (Fungicide Resistance Action Committee, FRAC, 2021). In parallel to the rapid evolution to resist fungicides, *Z. tritici* has also surmounted most known resistance factors segregating among wheat cultivars (Brown et al. 2015). Association mapping in *Z. tritici* has recently revealed specific mutations underlying the gain of virulence on previously resistant wheat cultivars including cultivars carrying the resistance gene *Stb6* and others (Zhong et al. 2017; Meile et al. 2018; Singh, Karisto, et al. 2021). Recently, Amezrou et al. (*preprint available shortly*) identified an additional 58 candidate virulence genes based on association mapping on 12 wheat differential cultivars. The genes linked to gains of virulence are typically referred to as effector genes and show rapid evolutionary change in populations of *Z. tritici* (Hartmann et al. 2017; Meile et al. 2018; Singh, Karisto, et al. 2021). Gene flow among *Z. tritici* populations is leading to significant weak differentiation at the continental scale and high local diversity (Linde et al. 2002; Zhan et al. 2003; Singh, Karisto, et al. 2021). Monitoring of fungicide resistance mutations is mainly achieved through the sequencing of target genes including the recent development of long-read sequencing assays (Samils et al. 2021). A joint monitoring of virulence mutations and genetic diversity is lacking though.

Here, we report the design and validation of a microfluidics based multiplex targeted amplicon sequencing assay that allows the simultaneous monitoring of mutations in fungicide resistance genes and effector genes associated with a wide range of host resistance factors. In addition, we enable the monitoring of hundreds of equally spaced polymorphisms along chromosomes to identify recent changes in the genetic composition of pathogen populations. We validate the performance of the assay using replication, sensitivity analyses to low input DNA, mixed samples as well as the performance on DNA directly obtained from infected wheat leaves.

Results

Marker design based on whole-genome sequenced individuals across species

We used whole-genome sequencing datasets of 632 *Z. tritici* isolates collected in Oceania (Australia, New Zealand), the United States, Switzerland, France, and Israel to identify segregating SNPs and improve the design of a total of 798 amplicons. **G**enome-wide polymorphism was used to mask sites to avoid primer mismatches and amplification drop-outs (Fig. 1A). We designed 25 amplicons across genes associated with fungicide targets and resistance including *CYP51*, *alternative oxidase* (*AOX*), *beta-tubulin* (*TUB1*) and *SDH1-4* genes including *ZtSDHC3* as well as *Cytochrome b (CYTB)* gene (See Supplementary Table S2). For each gene, we prioritized amplicons covering non-synonymous substitution. Due to the complexity of the transposable element insertion polymorphism in the promoter region of the transporter gene *MFS1*, we designed multiple amplicons matching known sequence variants near three insertion sites (Omrane et al. 2017) (See Supplementary Table S2). For loci associated with virulence on diverse cultivars, we retained a set of 67 amplicons successfully passing primer design (See Supplementary Table S2). We also randomly selected SNPs at ~50 kb distances including a SNP in the previously selected fungicide resistance gene *cytochrome b (CYTB)* to monitor the genetic make-up of populations for a total of 691 designed amplicons across all chromosomes (See Supplementary Table S2).

Assessment of loci quality across the targeted sequencing assay

We performed targeted sequencing of all 798 loci based on the Fluidigm Juno system in a single run using microfluidics (Fig. 1B). The 192 samples included four sets of pure DNA from different isolates mixed in equal proportions, ten samples including each DNA of the same three isolates in different proportions, and 178 samples constituted from extracted leaf material from different wheat fields across France mostly (*i.e.* n = 172), Belgium, Ireland and the United Kingdom. The complete set of samples was replicated once for the amplification and Illumina sequencing step. The total sequencing output over both replicates was 2,418,905,407 read pairs and 338.89 Gb. For 31 samples, the amplification and Illumina sequencing procedures failed in either one of the two replicates of each sample, therefore the failed replicates were eliminated. Across a replicate run (*i.e.* FC2), samples produced between 5,976-173,049,611 read pairs with numbers broadly consistent between the two replicate runs (Fig. 2A).

We found that the mapping rate against the reference genome ranged from 96.93-100% among all replicates of samples (Fig. 2B).

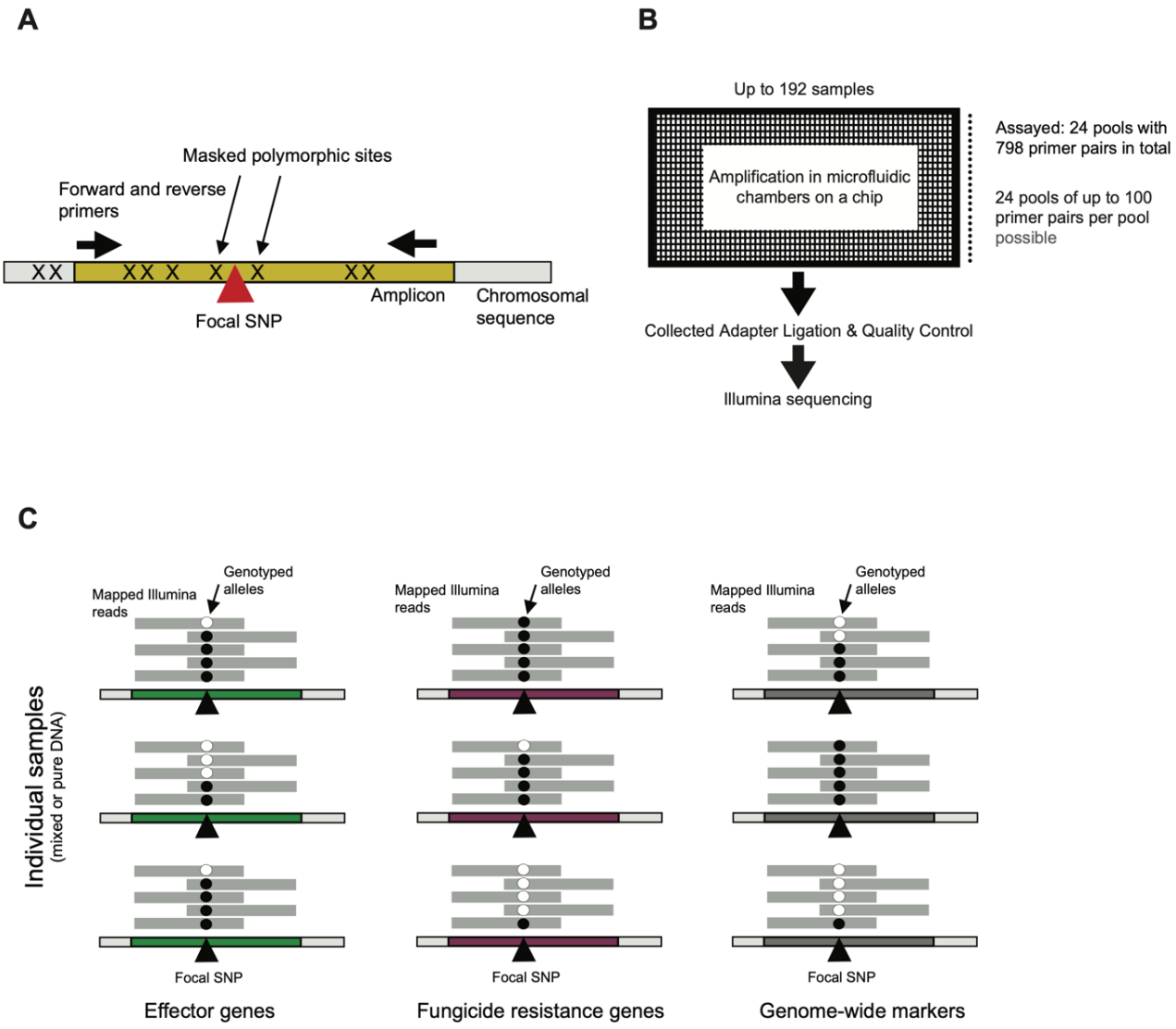

Figure 1: Schematic overview of the targeted amplicon assay design. A) Design of individual amplicons (~200 bp) with primers designed to not overlap known polymorphic sites. B) Schematic overview of the microfluidic chambers of a Fluidigm Juno chip accommodating up to 192 samples and 24 pools of primers (each up to 100 primer pairs). Following amplification in microfluidic wells, barcoded products are pooled and finalized for Illumina sequencing. C) Genotypes of individual samples (pure or mixed individuals) are assessed by analyzing mapped reads at each locus in the genome. Markers were designed for three different categories including effector genes, genes encoding targets of fungicides and genome-wide evenly spaced markers.

To assess the faithful amplification of individual loci, we first focused on the four samples with mixtures of pure fungal DNA of 26 to 30 isolates. Combining the two replicates, we used eight samples to evaluate sequencing read coverage across the 782 amplicons designed outside of the *MFS1* region. We found 17 loci with a read depth of 0. The highest read depth was 1,779,927 for an effector locus on chromosome 1. For the set of genome-wide, equally spaced amplicons on core chromosomes, we retained the locus if the read counts were between 20,000 and 100,000 in the set of reference samples (Fig. 2C). We considered this read count range to reflect the loci consistently amplifying across samples and not showing evidence for duplications. With this filter, we discarded 149 loci falling outside of the read count range (Fig. 2D). For randomly selected markers on accessory chromosomes, we expected lower amplification success because not all isolates of the species carry the locus. We retained loci with a read count between 10,000 and 100,000 in the set of reference samples leading to the rejection of 21 loci (Fig. 2C-D). For randomly selected mitochondrial markers, we found read counts ranging from 203,502 to 1,372,965 in the set of reference samples reflecting the high copy number of mitochondria compared to the nuclear genome. All 12 randomly selected mitochondrial loci were kept. For effector loci, the number of mapped reads ranged from 502 to 1,779,927 reads indicating significant variation in the amplification success and possibly copy number (Fig. 2C). We retained all 67 designed amplicons due to the general interest in polymorphism at such loci (Fig. 2D). For resistance gene loci, the number of mapped reads ranged from 2,986-1,372,965 reads (Fig. 2C). As for effector gene loci, all 24 designed amplicons were retained (Fig. 2D). In addition, we retained the amplicon for the mitochondrial resistance locus of *CYTB* with a read count of 1,372,965. In summary, we retained 521 high-quality loci representing 75% of the randomly selected markers designed for genetic structure analyses, as well as all 67 effector and 24 fungicide resistance loci (Fig. 2D).

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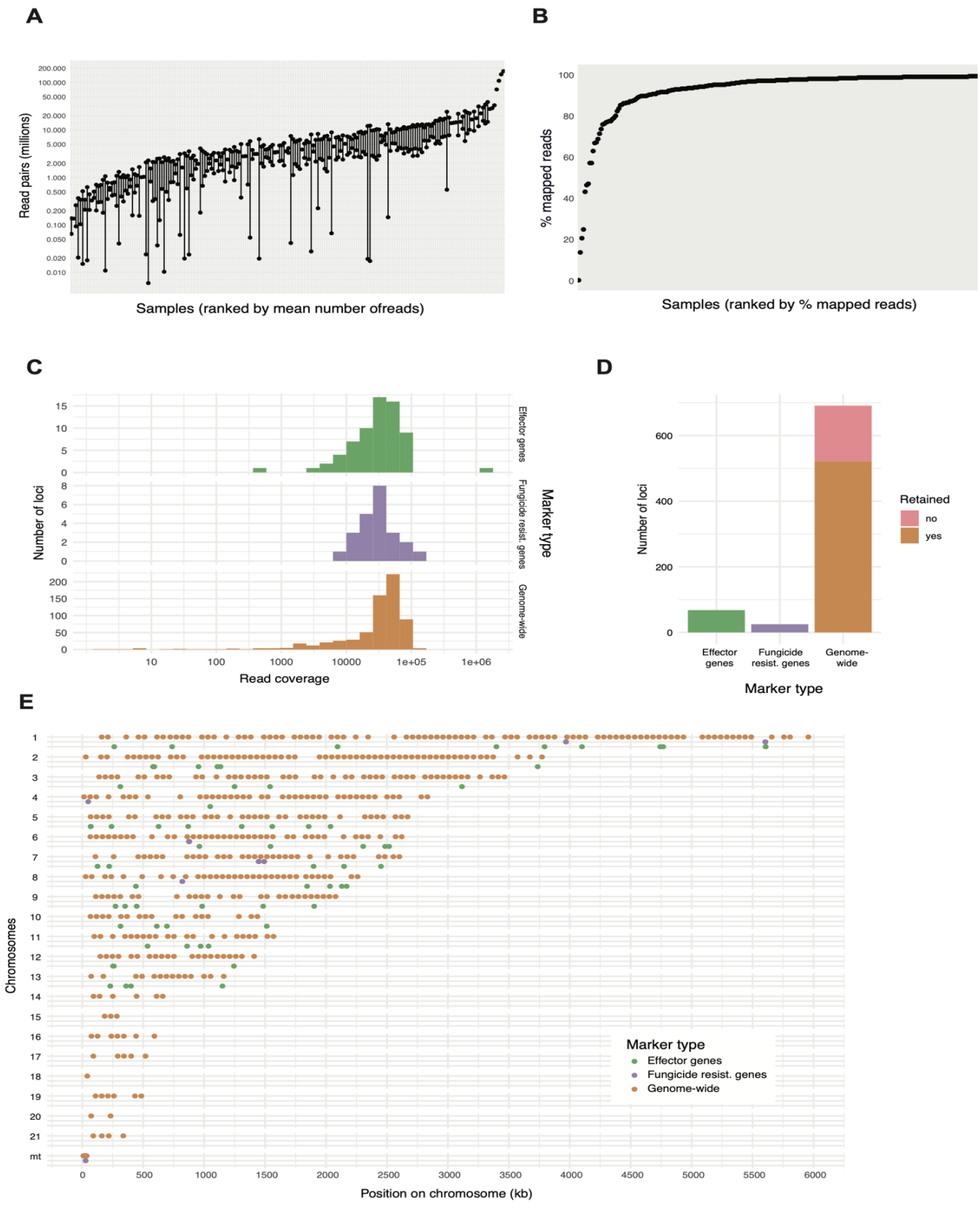

Figure 2: Sequencing data recovered for the amplicon assay and loci assessment. A) Read pairs recovered per sample and replicate. Each sample was amplified and sequenced two times (two different microfluidic flow cells). B) Ranking of percent mapped reads to the reference genome per sample (including both replicates if available). C) Number of reads mapped per locus for the three different categories of markers. The read numbers correspond to the total obtained from four pooled samples performed in replicates. D) Summary of loci retained after read number filtering. Only genome-wide markers were removed if they failed filtering criteria. E) Overview of retained markers per category across the 21 chromosomes and mitochondrion.

Reproducibility among replicate assays and recovery of allele frequencies

To assess the reproducibility of the sequencing assay, we repeated the amplification and sequencing procedure two times. We found that the number of read pairs recovered for each sample were positively correlated between replicates ($r = 0.78$, p-value < 0.0001; Fig. 3A). We also found a positive correlation in the mapping rate of reads recovered from the same samples ($r = 0.85$, p-value < 0.0001; Fig. 3B). To investigate effects on allele frequencies assessed for mixed samples, we compared the pooled DNA of population 41 sample. We used allele frequencies estimated from read depth for the reference and alternative allele at SNP loci. Reference allele frequencies at 201 SNP loci calculated in both replicates of each sample were highly correlated ($r = 0.89$, p-value < 0.0001) with outliers corresponding to poorly covered loci in either one of the two replicates of the same sample (Fig. 4A). Furthermore, we analyzed allele frequencies in 10 samples constituted from a mix of pure DNA from the same three isolates in different proportions. We focused on the effector locus *AvrStb6* on chromosome 5 with known differences among the three isolates. We found that the allele frequency variation at the SNP position 69,631 among samples with different isolate DNA mixtures was reflecting differences in the proportion of DNA mixtures (Fig. 4B). We analyzed correlations between the proportion of DNA of a specific isolate and allele frequencies (Fig. 4C; Supplementary Figure S2). As expected, based on the known genotypes, increasing proportions of DNA from isolate INRA10-FS1006 tended to be correlated with higher alternative allele frequencies (Fig. 4C). Increasing proportions of DNA from isolate INRA10-FS1022 tended to be correlated with lower alternative allele frequencies (Supplementary Figure S2B). However, neither correlation was significant.

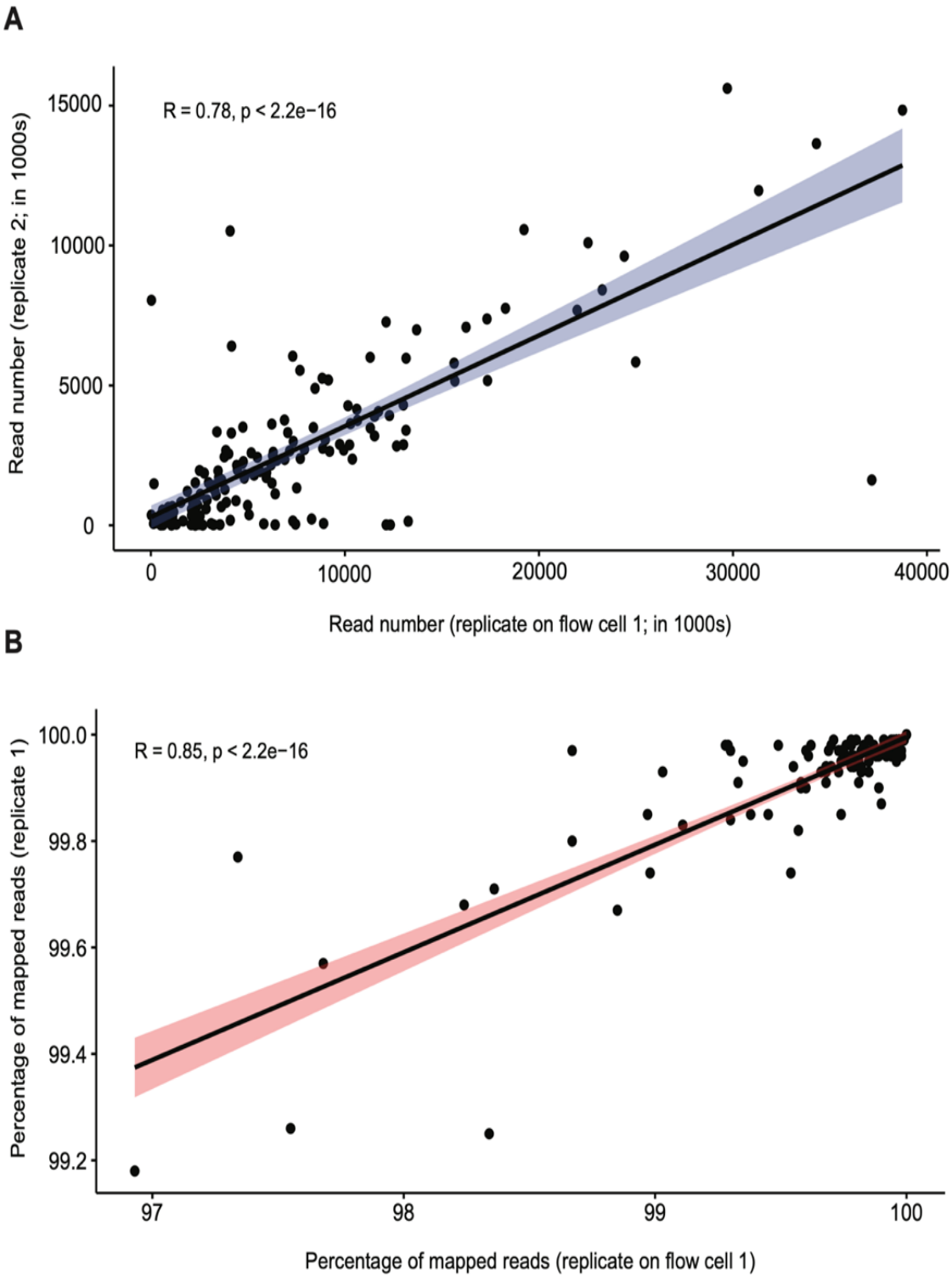

Figure 3: Consistency between replicate runs of the amplicon assay. A) Read numbers per sample and B) percentage of reads mapped to the reference genome.

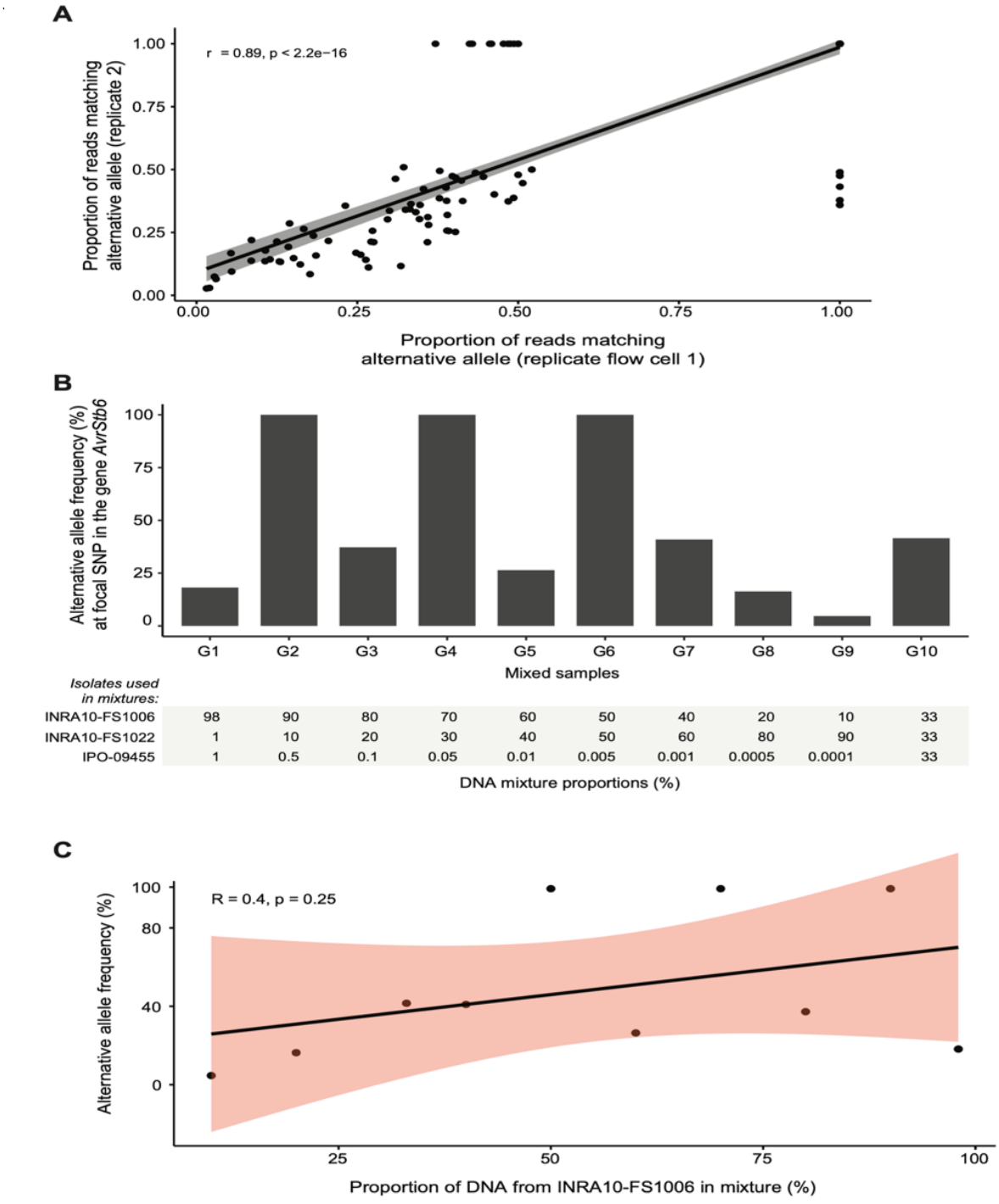

Figure 4: Evaluation of mixed sample analyses. A) Comparison of alternative allele frequencies within samples between the two replicate runs for each sample. B) Comparison of alternative allele frequencies within DNA mixture samples containing different proportions of three isolates. C) Correlation of proportion of the isolate INRA10-FS1006 compared to the alternative allele frequency. *Amplicons for the promoter region of MFS1*

The amplicons designed for the promoter region of *MFS1* are matching known haplotypes differing in their insertion of transposable element sequences. Due to the sequence complexity, we chose to first cluster sequencing reads into individual amplicons instead of directly mapping reads to a *MFS1* haplotype. Analyzing the 10 samples with different DNA mixtures of three isolates including replicates, we identified 10 sequence clusters with at least 22 reads (lowest number observed in sample G3). We used BLAST to retrieve the subset (n =10) of the clustered sequences matching the *MFS1* promoter region. The sequences matched positions from 1-4946 bp (for sample G9) on the consensus *MFS1* sequence with all being upstream of the coding sequence as expected (Fig. 5A). We did not recover any amplicon matching forward and/or reverse primer positions based on the amplicon design (Fig. 5B). However, all amplicons did not match the expected amplicon length most likely due to the complexity of the underlying sequence. Furthermore, the pooled amplification of multiple primer pairs matching the promoter region has likely produced chimeric amplicons in some contexts. We used the retrieved amplicons matching the promoter region to form clusters of near identical BLAST matches based on alignment length and positions. We identified 10 well supported amplicon clusters showing variation in abundance among the analyzed samples (Supplementary Table S3).

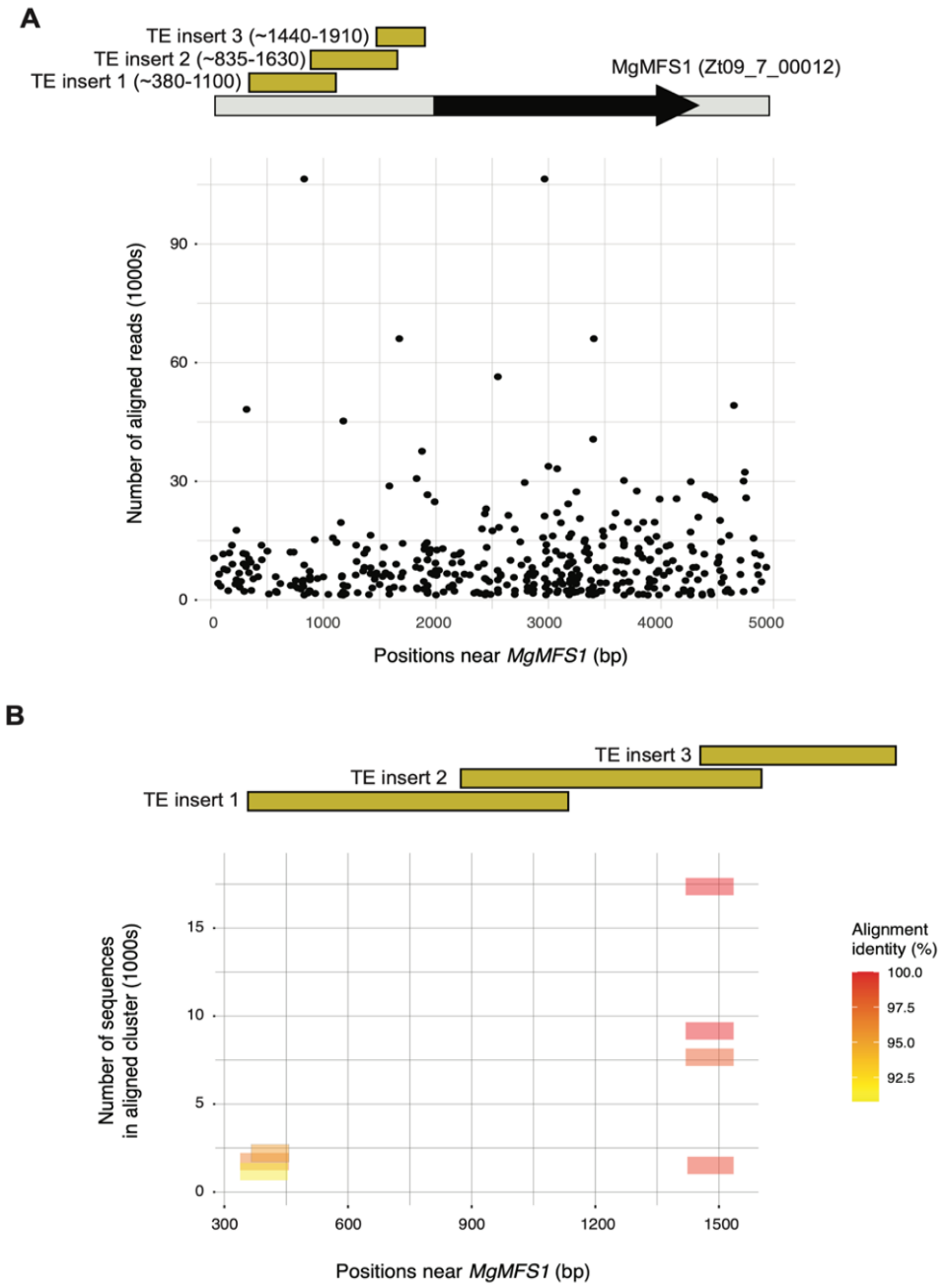

Figure 5: Analyses of amplicons designed on polymorphic transposable element insertions upstream of the multidrug transporter gene *MgMFS1*. A) Overview of the location of amplicons designed for each of three transposable element insertion site (1-3). Multiple amplicons were designed for each insertion site. The aligned reads are shown for positions near the coding sequence of *MgMFS1* for sample G9 (only positions with >10 reads mapping are shown). B) After read clustering for sample G9, consensus sequences were blasted against positions near the coding sequence of *MgMFS1*. The horizontal bars indicate the extent of a BLASTn alignment with colors indicating the percent identity of the alignment. The vertical position indicates the number of sequences that were clustered for the aligned consensus sequence.

Genetic differentiation in French and European wheat field populations

We used the 158 wheat leaf samples infected by *Z. tritici* collected from fields across France with at least five genotyped samples per location and additional samples from Belgium, Ireland and the United Kingdom to assess the genetic structure using the genome-wide marker set. Based on a principal component analysis of 85 genome-wide SNPs, we found that the majority of the European samples grouped homogeneously (Fig. 6A). Focusing on the genetic differentiation among French regions (n = 82 genome-wide SNPs for the French populations only), we found some modest differentiation of genotypes from wheat fields in Midi-Pyrénées and Champagne (Fig. 6B). However, the overall differentiation of the field samples was low with the first and second principal component explaining only ~4%.

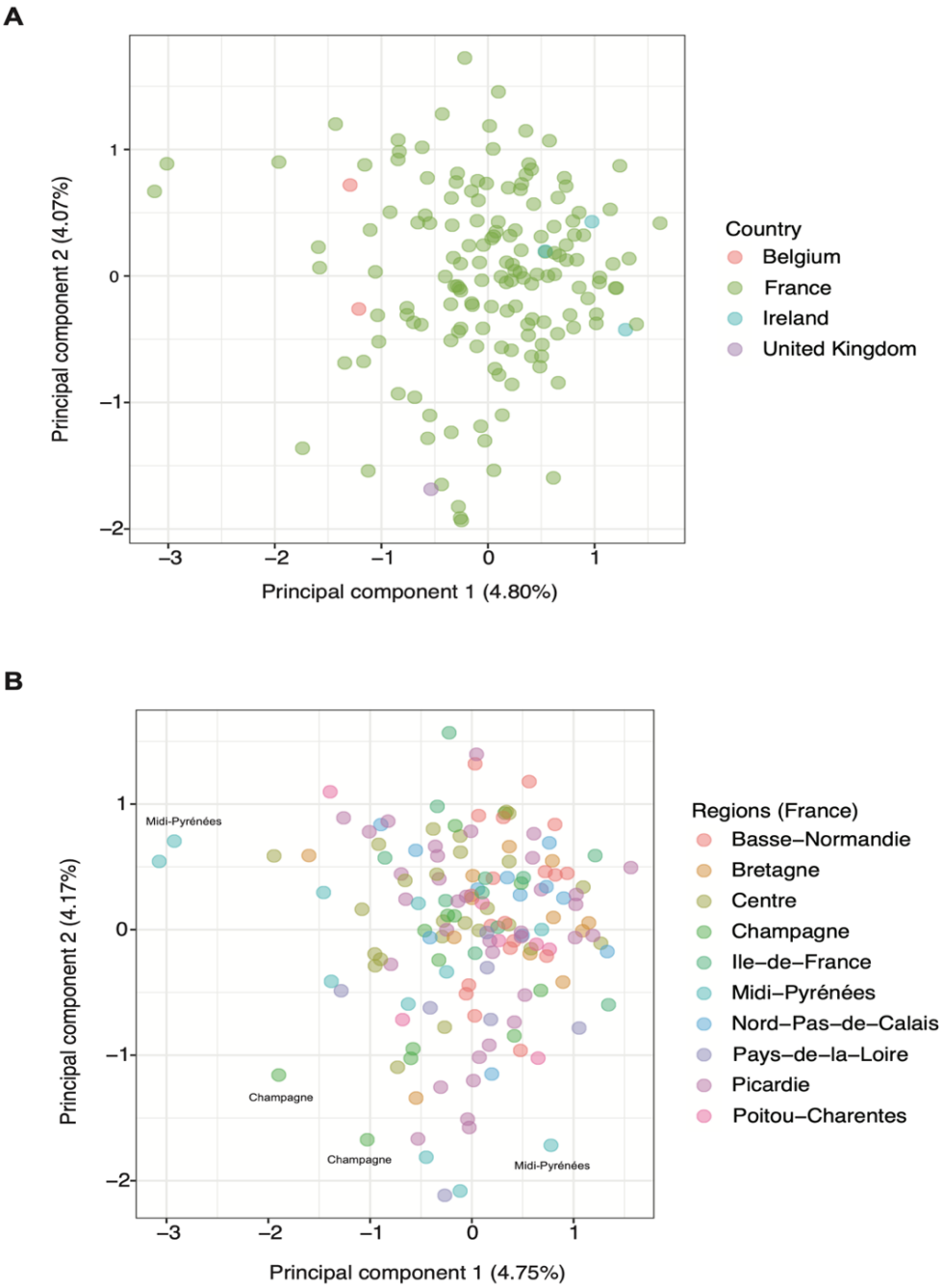

Figure 6: Population structure analyses based on genome-wide markers genotyped on leaf-extracted assemblies of *Zymoseptoria tritici* strains. A) Principal component analysis of wheat leaf samples collected in France, Belgium, Ireland and the United Kingdom and B) the subset of wheat leaf samples collected in France colored by region.

Discussion

We developed a microfluidics-based amplicon sequencing assay combining the advantages of high-throughput sequencing and multiplex PCR. We assessed the performance of 798 loci to reliably and sensitively genotype randomly selected genome-wide markers, as well as virulence and fungicide resistance genes in a diverse set of *Z. tritici* samples. We show that a large portion of the designed markers can be amplified consistently across samples and used to monitor the emergence of relevant mutations. The set of genome-wide markers provides means to assess the genetic structure of the pathogen directly from field collected wheat leaves.

Within-species polymorphism can lead to amplification failures due to mismatching primers. We considered this issue particularly relevant for the wheat pathogen *Z. tritici* as the species harbors genetically highly diverse populations within single fields (Singh, Karisto, et al. 2021). As expected, we detected a high number of SNPs in regions intended for amplicon design leading to the rejection of amplicon candidates prior to the genotyping stage. Furthermore, we noticed targeted regions with weak amplification success. The poor performance of some primer pairs is most likely explained by a combination of factors. First, we ignored low-frequency SNPs at the masking stage to be able to proceed to the amplicon design for more loci. Second, our species-wide genomic survey of SNPs may have missed polymorphisms present in the assayed samples. The filtering thresholds can be adjusted and more genome sequencing datasets could be included in future amplicon design efforts. Despite some failed attempts at amplifying individual loci, we obtained high degrees of sequencing read coverage for most loci. Most samples yielded hundreds to thousands of reads for each locus. Such deep coverage across the amplicon assay provides a detailed picture of genotypic diversity particularly for mixed samples directly obtained from infected leaves. A major limitation with the multiplexed amplicon sequencing assay is the shortness of the amplified sequence (~200 bp). The short amplicon length ensures a high degree of multiplexing by providing stable amplification across the entire assay. However, longer amplicons would be needed to recover entire sequences (*i.e.* haplotypes) of the azole resistance locus *CYP51* or several effector genes of interest. A potential solution would be to design

overlapping amplicons to cover an entire locus. However, this approach was unsuccessful *e.g.* for the effector gene *AvrStb6* providing no sufficiently conserved sections inside or adjacent to the coding sequence for an overlapping amplicon design.

A versatile pathogen genotyping assay should perform well with low-input pure fungal DNA as well as mixed samples containing DNA both from multiple strain genotypes and the host (*i.e.* wheat plants). We find that the assay replicated well across all tested sample types both in terms of the number of recovered reads per sample as well as the proportion of reads that could be mapped to the *Z. tritici* reference genome. We found that in mixed samples (*i.e.* containing more than one genotype), the assay reproduces well the allele frequencies across the two independent genotyping runs. We also assessed the ability of the assay to recover allele frequencies at a specific locus (*i.e.* the effector gene *AvrStb6*) using three-isolate mixtures of different proportions. We found a generally positive correlation of the proportion of a specific isolate in the DNA mixture and the allele associated with the isolate, however the recovery of allele frequencies was not well reproduced across all tested DNA mixtures. Hence, the assay requires further validation if within-sample allele frequencies need to be recovered with high accuracy. The genotyping of TE insertions in the *MFS1* promoter region was not conclusive. The overlapping amplicons and very high levels of sequence polymorphism prevented a clear assignment of amplicons to TE insertion genotypes, but our data opens up a path for a more comprehensive design strategy to capture inserted sequences. Overall, the assay performed well in recovering *Z. tritici* DNA across hundreds of loci even from wheat samples obtained directly from the field.

The microfluidics-based multiplex PCR targeted amplicon sequencing recovered successful genotypes at hundreds of targeted loci. The number of recovered loci for targeted amplicon sequencing remains below untargeted approaches such as RAD-seq and GBS. Untargeted reduced-representation approaches provide however only genome-wide information on genetic differentiation. This may be informative *e.g.* for virulence profiles in clonal pathogens (Aoun et al. 2020), however this approach is unsuitable to recover genotypes at specific loci. Targeted amplification such as the microfluidics based multiplex PCR performs also well in mixed samples. RAD-seq and GBS are unlikely to perform well

if substantial proportions of plant DNA are present, because large plant genomes will typically contain many more restriction sites compared to the fungal genome. Limitations in amplicon length can be overcome using long-read sequencing as developed to monitor fungicide resistance loci in *Z. tritici* (Samils et al. 2021). Long-read sequencing may also help to overcome issues with amplifying the highly polymorphic promoter region of *MFS1*. Long amplicons could capture the entire promoter region instead of focusing on individual insertion points. Ultimately, a combination of different approaches performing highly multiplexed short reads sequencing and separate long-read sequencing for the most complex loci will be required.

The developed microfluidics-based targeted amplicon assay allows a cost-effective and reproducible monitoring of hundreds of loci to track virulence and fungicide resistance evolution in field populations. The integration of genome-wide markers greatly enhances the information provided, compared to monitoring focused on adaptive loci alone, by providing crucial information about patterns of gene flow. Our study revealed only weak differentiation across Western European countries and among French regions consistent with high levels of gene flow and genetic diversity. Knowledge of genetic structure can help identify recent movements of the pathogen due to natural or human-mediated dispersal. The rapid rise in resistance of *Z. tritici* populations after the application of fungicides can more effectively be monitored due to the large number of loci that can be assayed simultaneously. Furthermore, tracking mutations at effector loci opens new opportunities to track adaptation to different wheat cultivars across regions. With the availability of whole genome sequencing data for an increasing number of crop pathogens, the targeted amplicons could be expanded to simultaneously or separately genotype other major pathogens including rusts to improve the surveillance and management of crop diseases globally.

Methods

Isolates used for the design and validation of the assay

We retrieved genome sequencing information from 632 *Z. tritici* isolates collected across the global distribution range of wheat. Isolates included six different populations with a sample size of 29-178. A total of 88 isolates were collected in Australia including Tasmania in 2001 and 2015 (Hartmann et al. 2017). Additional isolates from Oceania included 75 isolates collected in New Zealand in 2013 and 2015 (Feurtey, McDonald, et al. 2022). A total of 154 isolates were collected in Oregon, USA, in 1990 and 2015 (Hartmann et al. 2017). 178 isolates were in wheat fields near Zurich in Switzerland in 1999 and 2016 (Hartmann et al. 2017; Singh, Karisto, et al. 2021) and 29 isolates were isolated in the Nahal Oz region in Israel in 1992 (Hartmann et al. 2017). Finally, 108 isolates were retrieved from a panel of French isolates (Zhong et al. 2017). We assessed the performance of the microfluidics assay using different sets of samples collected from wheat fields in Europe. Four samples included equimolar DNA mixtures of 26 to 30 isolates obtained by culturing single spore isolates from field-collected wheat leaves. Three single spore isolates identified as INRA10-FS1006, INRA10-FS1022 and IPO-09455 were collected in 2009 and 2010 in the Ile-de-France region and were used to create DNA mixtures in ten different proportions. Finally, a total of 178 samples were obtained by extracting DNA directly from infected wheat leaves collected in different regions of France, Belgium, Ireland and United Kingdom. See Supplementary Table S1 for details on all included samples.

SNP calling and identification of polymorphisms for the amplicon design

We performed read alignment and SNP discovery for the generated genomic datasets, as previously described (Hartmann et al. 2017; Zhong et al. 2017). In summary, we trimmed raw Illumina reads using Trimmomatic v. 0.38 (Bolger et al. 2014) and mapped retained reads to the reference genome IPO323 (Goodwin et al. 2011) using bowtie v2.3.5 (Langmead and Salzberg 2012). We used the Genome Analysis Toolkit (GATK) v4.0.1 (Van der Auwera et al. 2013) including the HaplotypeCaller tool to identify candidate SNPs. We filtered for a set of high-quality polymorphisms using the GATK

VariantFiltration tool and vcftools v.0.1.15 (Danecek et al. 2011). A more extensive description of the filtering procedures and validations are available (Hartmann et al. 2018).

Polymorphism selection for neutral markers, effector and fungicide resistance genes

Effector candidate genes were retrieved from GWAS focused to identify candidate effectors interacting with major wheat resistance genes (Amezrou et al., unpublished, Hartmann et al. 2017, Meile et al. 2018). We included 65 candidate effector genes showing a significant association for symptom development on at least one wheat cultivar. We designed at least one amplicon overlapping the most significantly associated SNP in each of the effector genes. If a significantly associated SNP could not be reproduced in the worldwide isolate collection, a random nearby SNP (within ~200 bp) was selected as the target for the amplicon design. If a different SNP was selected, we filtered for SNPs with a minor allele count of 5 and a minimal genotyping rate of 80%. For the effector gene *AvrStb6,* we designed two additional amplicons to cover polymorphism in the coding sequence. To monitor fungicide resistance gene mutations, we covered 25 genes related to fungicide resistance in *Z. tritici* populations including the mitochondrial gene *CYTB* associated with resistance to strobilurin (QoI)*,* the nuclear genes beta tubulin 1 (carbendazim resistance)*, CYP51* (azole resistance)*, AOX* (alternate oxidase inhibitor resistance), as well as *SDH1, SDH2, SDH3* and *SDH4* (SDHI resistance). The amplicons covered known resistance mutations if known. If no mutation was documented in *Z. tritici,* the amplicon covered randomly selected SNPs in the coding sequence. Similar to the procedure for effector loci, if a known SNP associated with fungicide resistance could not be recovered, we selected a SNP within ~100 bp (minor allele count of 3, minimum genotyping rate 80%). The broader inclusion of polymorphisms for filtering was possible due to the generally lower degree of detected variants in resistance genes. We defined an additional amplicon to target the paralog of *SDH3* (*ZtSDHC3*; Steinhauer et al., 2019). For this, we analyzed the paralog sequence discovered in the pangenome of *Z. tritici* (Badet et al. 2020). Multidrug fungicide resistance in *Z. tritici* is mediated by transposable element (TE) insertions in the promotor region of the transporter *MFS1*. We designed 16 amplicons covering three previously reported TE insertions and haplotypes (Omrane et al. 2017). The amplicons were designed to either amplify if an insertion was present or not. Amplicons were designed on a

consensus sequence of previously described haplotypes (Omrane et al. 2017). In addition to polymorphisms related to virulence and fungicide resistance, we randomly selected equally spaced polymorphisms along all 21 chromosomes to capture neutral population structure. For this, we selected 691 SNPs with a minor allele frequency of 5% and a minimal genotyping rate of 80%. SNPs were selected at a distance of 50 kb (if available) using the *–thin* option in *vcftools*. In conclusion, a total of 798 amplicons were designed for virulence, fungicide resistance as well as gene flow tracking across *Z.tritici* populations. See Supplementary Table S2 for details on all selected effector and fungicide resistance genes as well as whole genome neutral markers.

Amplicon design

For genome-wide markers and markers in virulence and fungicide resistance genes (except *ZtSDHC3* and MFS1), we produced a 401 bp fasta sequence from the reference genome centered on the SNP to target. We identified the SNP using the IUPAC code marked with parenthesis according to the design instructions. To improve genotyping success across a broad range of *Z. tritici* genotypes, we masked known polymorphic sites near the targeted SNP to prevent the accidental primer design in known polymorphic regions. We used bcftools v1.9 (Li 2011) to mask non-target sites showing evidence for polymorphisms in the panel of 632 analyzed isolates using the -I option of the consensus command and re-wrote sequences with samtools v1.9 (Li et al. 2009). For resistance and effector genes, we used a minor allele count of 3 and 5, respectively to consider the polymorphism for masking. For genome-wide markers, we used a minor allele frequency cut-off of 5%. If the resulting sequence contained more than 10% masked sites, the amplicon was not considered further. Additional sequences were excluded by Fluidigm Inc. if the sequences failed to yield adequate primer candidates. If the initial amplicon design had failed, we repeated the procedure for effector loci but relaxed the filter to consider only SNPs with a minor allele count of ≥25.

DNA extractions and microfluidics assay

DNA extractions to test the microfluidics assay were performed using the following procedures. For pure cultures and directly from infected wheat leaves using Dneasy® Plant Mini Kit (Qiagen, Hilden, Germany). DNA was quantified using a Qubit 2.0 fluorometer (Thermo Fisher, Waltham, Massachusetts, USA). We followed the Fluidigm Inc. (San Francisco, California, USA) Juno^{TM} targeted amplicon sequencing protocol according to the manufacturer's protocol. As input DNA, we used the following 1.5-200 ng of total amount (See Supplementary Table S1). We performed the entire microfluidics procedure twice independently on different Juno LP 192.24 integrated fluidic circuits plate (IFC). Libraries were prepared following the manufacturer's protocol. Target amplicons were generated for each sample and pools of primers using PCR on a specialized thermocycler (Juno system; Fluidigm). Illumina sequencing was performed in paired-end mode to generate 100 bp reads on the NovaSeq™6000 platform at Integragen Inc. (Evry, France) and produced 363.89 Gb of raw sequencing data for both independent chips combined.

Amplicon sequence data analyses

We used Trimmomatic v0.38 (Bolger et al. 2014) with the following parameters: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36. Due to the short amplicon length compared to the read lengths, we used FLASH v1.2.11 (Magoč and Salzberg 2011) to merge forward and reverse reads per pair into single pseudo-reads. Finally, pseudo-reads were aligned to the IPO323 reference genome using bowtie2 v2.3.5 (Goodwin et al. 2011; Langmead and Salzberg 2012). We assessed individual read counts at each analysis step using MultiQC v.1.7 (Ewels et al. 2016). After individual genotyping using the GATK HaplotypeCaller tool, we performed multi-sample genotype calling using CombineGVCFs and GenotypeGVCFs (Van der Auwera and O'Connor 2020). Variant sites were removed if these met the following conditions: QD < 5, MQ < 20, -2 > ReadPosRankSum > 2, -2 > MQRankSum > 2, -2 > BaseQRankSum > 2. For amplicons targeting the promoter region of *MFS1*, we first used seqtk (Shen et al. 2016) to subsample 10.000.000 reads from large merged paired-end reads FASTQ files and we then performed a clustering analysis of Illumina reads to obtain read sets originating from the same locus. We used CD-HIT-EST (Fu et al. 2012) with an identity threshold set to 100% to cluster sequencing reads. For each cluster, the representative sequence identified by CD-

HIT-EST was aligned to the *MFS1* promoter consensus sequence using BLASTn 2.12.0 (Altschul et al. 1990). Only BLASTn best hits with a bit score above 100 and identity > 90% were kept. To identify clusters of nearly identical hits based on position and identity, we performed *k*-means clustering with the R packages {factoextra}(Kassambara and Mundt 2020), {clustertend} (Wright et al. 2022), {cluster} (Maechler et al. 2012), {NbClust} (Charrad et al. 2014). For each sample, we identified the optimal number of clusters (K = 1–10) by performing a silhouette analysis (Rousseeuw 1987).

Data visualization and population genetic analyses

Data analyses were performed using R 4.0.4 (R Core Team 2021a). The R packages included in {tidyverse} (Wickham et al. 2019) were used for summarizing and plotting coverage across loci, visualizing retained SNPs, the outcomes of different filtering stages and genotyping. We used bcftools v1.9 (Li 2011) to calculate allele frequencies at SNP loci. The allele frequency correlation between both flow cells chips was analyzed with the R package {report} (Makowski et al. 2021) and visualized using {ggpubr} (Kassambara 2020) and {ggplot2} (Wickham 2016). To analyze genetic diversity and population structure, we performed a principal component analysis (PCA) using the R packages {vcfR} (Knaus and Grünwald 2017), {adegenet}(Jombart and Ahmed 2011), {ade4} (Dray and Dufour 2007) and {ggplot2} (Wickham 2016). For population analyses, we focused only on the second replicate (flow cell) and genome-wide SNPs were filtered for a minor allele frequency of 0.05 and allowing for 20% missing data (--max-missing 0.8).

Data availability: Raw sequencing data is available on the NCBI Sequence Read Archive (SRA) under BioProject PRJNA847707.

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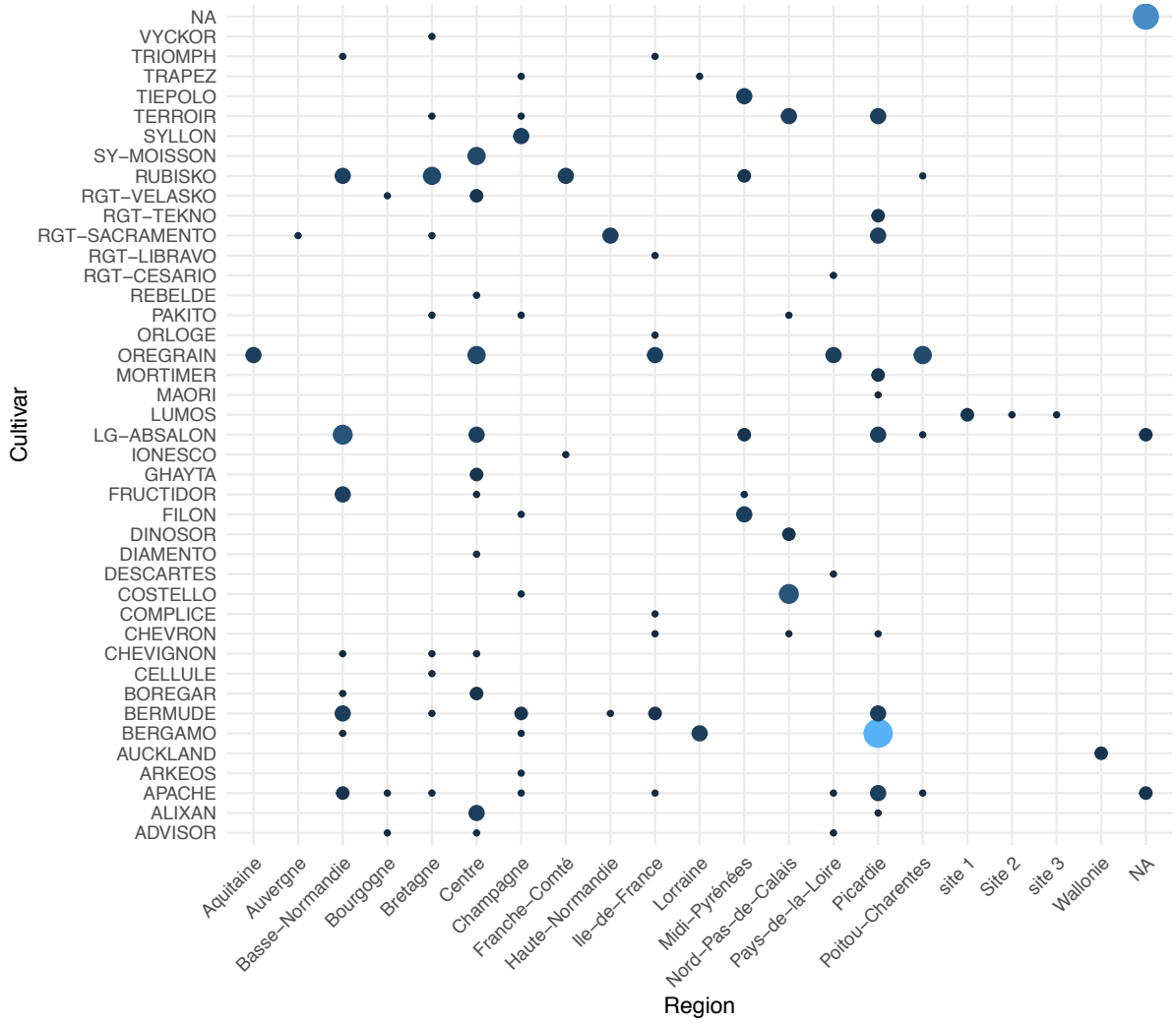
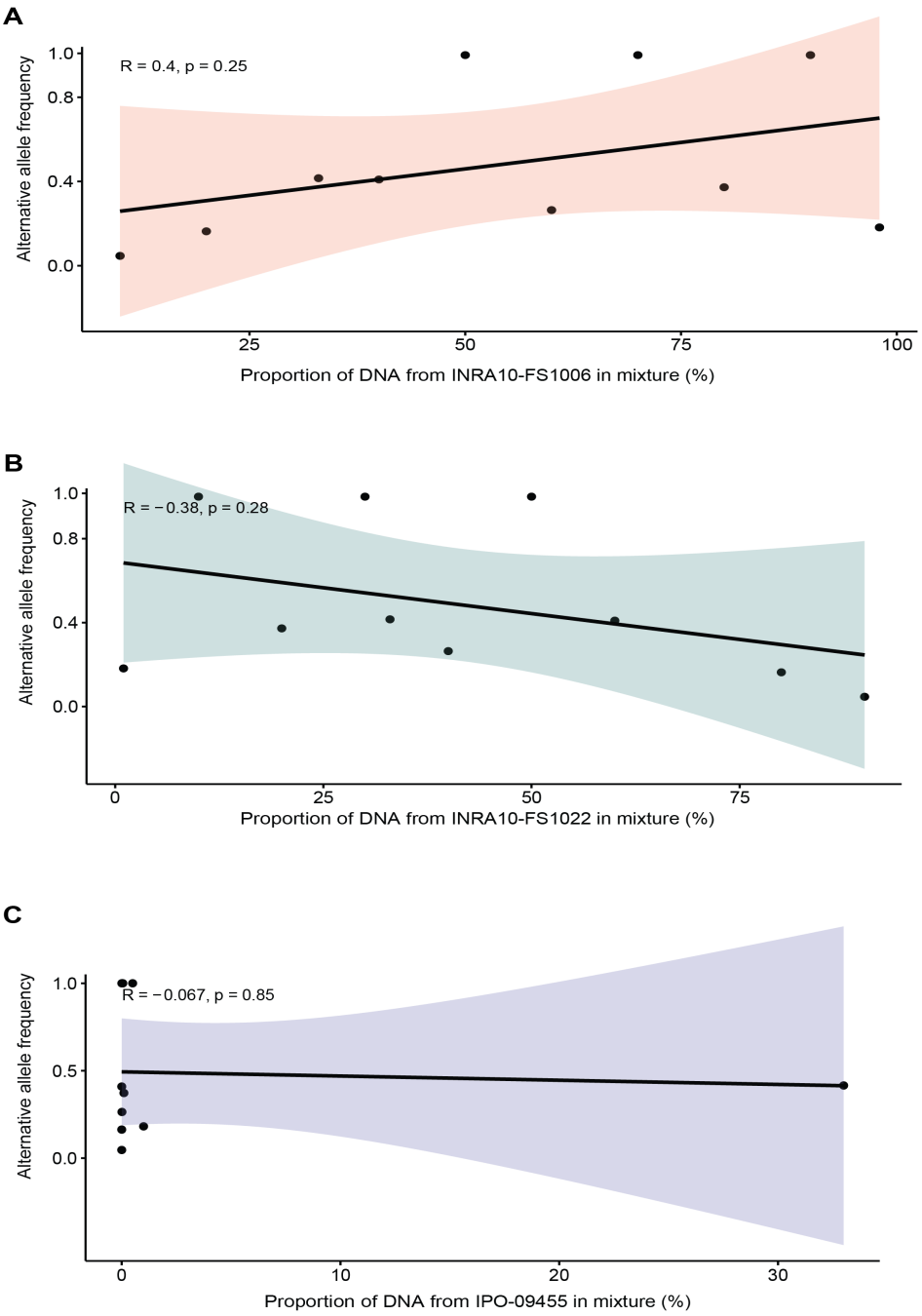
Supplementary information

Supplementary Figures



Supplementary Figure S1: Wheat leaf samples collected in France, Belgium, Ireland and the United Kingdom separated by the cultivar of origin or unknown cultivar ("NA"). See Supplementary Table 1 for details on the sample origins.



Supplementary Figure S2: Evaluation of mixed sample analyses A) Correlation of proportion of the isolate INRA10-FS1006 compared to the alternative allele frequency. Identical analyses for B) isolate INRA10-FS1022 and C) isolate IPO-09455.

Supplementary Table legends

(See Excel file)

Supplementary Table S1: Samples and sample mixtures included in the microfluidics assay.

Supplementary Table S2: Designed amplicons targeting neutral markers, fungicide resistance and effector genes. Loci check: coverage based assessment of amplification success (see methods). If an originally targeted locus was not recovered in the species-wide SNP call set used for the amplicon design, a nearby SNP was chosen (see last columns for newly selected loci).

Supplementary Table S3: Clustering of reads using CD-HIT-EST followed by mapping to the MFS1 promoter region. Similar blast hits were grouped into K-means based clusters.

Chapter 2

Divergent outcomes of direct conspecific pathogen strain competition and during plant co-infection with consequences for disease dynamics

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Abstract

Plant diseases are often caused by co-infections of multiple pathogens with the potential to aggravate disease severity. In genetically diverse pathogen species, co-infections can also be caused by multiple strains of the same species. However, the outcome of such mixed infections by different conspecific genotypes is poorly understood. The interaction among pathogen strains with complex lifestyles outside and inside of the host are likely shaped by diverse traits including growth rates, metabolism, and the ability to overcome host immune responses. To disentangle competitive outcomes among pathogen strains, we investigated the fungal wheat pathogen *Zymoseptoria tritici*. The pathogen infects wheat leaves in complex strain assemblies and highly diverse populations persist between growing seasons. Here, we investigated a set of 14 genetically different strains collected from the same field to assess in parallel competitive outcomes under culture conditions and on the host. We found that growth kinetics of co-cultured strains significantly deviated from single strain expectations indicating complex competitive exclusion depending on the strain genotype. We found similarly complex outcomes of lesion development on plant leaves following co-infections by the same pairs of strains. While some pairings suppressed overall damage to the host, other combinations have exceeded expected lesion development. Importantly, strain competition outcomes in absence of the host were poor predictors of outcomes inside the host showing that the interaction with the plant immune system adds significant complexity. In conclusion, our study shows how intraspecific co-infection dynamics make important contributions to disease severity and need to consider understanding host-pathogen dynamics.

Introduction

Plants are exposed to a variety of microorganisms in their natural environment (Brown et al. 2010; Anfoka et al. 2016; Xu et al. 2022). Colonizing microorganisms often occur as complex communities with complex interkingdom (mainly bacteria and fungi) interactions on single plant host (Salvaudon et al. 2013; Susi et al. 2015; Tollenaere et al. 2017; Ontiveros et al. 2022). Colonizing by multiple pathogenic organisms are knowns as coinfection and are very common in nature (Susi et al. 2015). Co-infections are of particular interest since the interaction between pathogens can significantly alter infection severity (Fang et al. 2021). Because co-infections can modulate the selection pressure acting on each pathogen, the evolution of pathogen virulence can have altered trajectories (Alizon et al. 2013; Tollenaere et al. 2016). Co-infecting pathogens can cause varying levels of damage to the host depending on the outcome of microbial interactions and host immune responses (Abdullah et al. 2017). For example, previous studies showed that when some plants are co-infected by pathogens of different species, one species tends to decrease the growth and development of the other competing species (Orton and Brown 2016). Moreover, co-infection has not always been associated with more severe disease symptoms than single infection (Abdullah et al., 2017).

The outcome of co-infections can be affected by the order of co-infection. Sequential co-infection can decrease the virulence of the second strain involved in the co-infection because of the priming of plant defenses (Aghnoum and Niks 2012). The host immune defenses activated by the first pathogen might not be mounted rapidly enough for the first pathogen, but the second infecting pathogen might be unable to colonize the host. Consistent with this observation, simultaneous infections can be more harmful to hosts than sequential infections (Marchetto and Power 2018). In several cases, co-infection can increase disease severity through pathogen cooperation. For example, microbial cooperation increased disease caused by the ascomycete *Didymella bryoniae* by helping translocating four bacterial species that co-infect Styrian oil pumpkin (Grube et al. 2011). Pathogen competition among individuals of the same species can also occur (Leggett et al. 2013) with conflicts over access to nutrients in well-defined niches such as plant leaves (Gold et al. 2009). Previous work shows that the interactions between co-infecting

pathogens are context-dependent with many factors influencing the outcome of infections and are, hence, difficult to predict.

For plants, intraspecific co-infections are playing a key role in disease progression with fungal-plant pathosystem receiving most attention (Schürch and Roy 2004; Susi et al. 2015; Barrett et al. 2021)

Plant pathogenic fungi display a wide range of specialization levels from generalists able to infect hundreds of host plants to specialists infectious only on single host species (Borah et al. 2018; Li, Cornelissen, et al. 2020; Newman and Derbyshire 2020). Within pathogen species, strains can show a high degree of variation in virulence (Schürch and Roy 2004; Barrett et al. 2021). The virulence of co-infecting *Podosphaera plantaginis* strains can be increased on the *Plantago lanceolata* host (Susi et al. 2015). In other systems such as *Zymoseptoria tritici* infecting wheat, virulence appears unaffected by co-infections (Schürch and Roy 2004; Barrett et al. 2021). However the trajectory of an infection depends on the host genotype and genotypes of competing strains (Barrett et al. 2021; Bernasconi et al. 2022). Besides, It has been shown that the competitive advantage of individual strains genotypes for transmission is not associated with virulence among the strains (Bernasconi et al. 2022). The growth of mixed genotypes outside of the host is only poorly associated with the success of the same genotype mix on the plant though (Bernasconi et al. 2022). The complex links between competitive abilities of the pathogen and consequences for the plant host raise important questions how co-infections impact evolutionary trajectories of host-pathogen systems.

Z. tritici is a major fungal pathogen of wheat causing the economically important foliar disease Septoria tritici blotch (STB) (Fones and Gurr 2015). *Z. tritici* exhibits a symptomless phase after coming into contact with wheat leaves lasting 8–11 days post infection (dpi), during which the fungus does not induce visible host defense responses (Sánchez-Vallet et al. 2015; Fagundes et al. 2020). In later stages the fungus invades the intercellular space of leaves producing lesions and asexual fruiting bodies called pycnidia. Under co-infection, *Z. tritici* strains colonize wheat leaves in a mosaic of genotypes (Bernasconi et al. 2022). Field populations exhibit high genetic and phenotypic diversity worldwide

due to frequent sexual reproduction, large populations, and significant gene flow among populations (Zhan et al. 2005). Co-infection of wheat leaves by multiple strains of *Z. tritici* are frequent in the field (Linde et al. 2002).

The aim of this study is to investigate the outcome of pathogen strain co-existence both outside of the host and on the host. For this, we analyzed 15 different strains sampled from the same wheat field and set up 91 and 105 different pairs to assess competitive outcomes among strains both inside and outside of the host respectively and the impact of co-infection on disease severity.

Results

In-vitro growth rate assessment and variation among strains

We analyzed the growth of 14 *Z. tritici* strains and 91 pairwise combinations in liquid growth medium (See Supplementary Figure S1; Supplementary Tables S1-2). In general, we observed stationary growth after 11 days of incubation at 18 °C (Fig. 1A). We assessed different culture growth metrics including the maximum specific growth rates *(μmax)* and doubling times. Maximum specific growth rates *(μmax)* and doubling times for different strains were highly correlated (Pearson correlation, $r = -0.96$, *p-value* < 0.0001; Fig. 1B). Single strains displayed variable maximum specific growth rates *(μmax)* (Fig. 1C). Analyzing the 91 pairs established for co-cultures, we found that in 37 pairings the strains showed a significant difference when grown independently (*p-value* < 0.05; Fig. 1D). Each of the 14 strains used for the pairings was showing a growth significantly different in at least one pairing (*p-value* < 0.05; Fig. 1D). The strain 3K8 showed significantly higher maximum specific growth rates *(μmax)* in 9 out of the 13 pairings (*p-value* < 0.05; Fig. 1D). Strain 1S10 was growing significantly faster in 6 out of 13 pairings (*p-value* < 0.05; Fig. 1D). In contrast, the strains 1G30, 1P35, 3R39, 3W21 and 3W3 were significantly slower growing compared to all their pairings (*p-value* < 0.05; Fig. 1D).

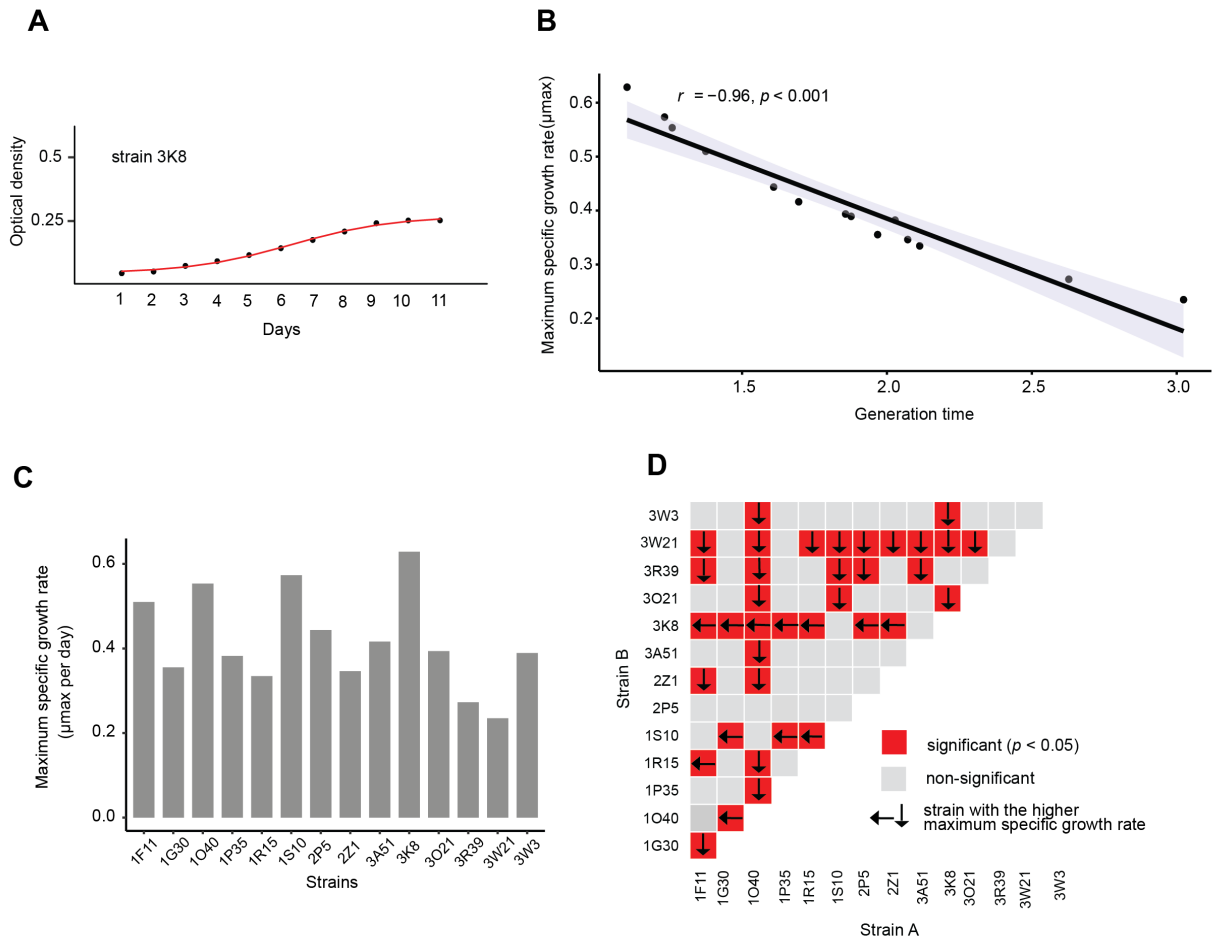

Figure 1: Overview of *Z. tritici* strains growth during in-vitro experimental conditions. A) *Z.tritici* strain's growth curve, stationary growth phase was reached after 11 days of incubation in dark at 18 °C. B) Intrinsic growth metrics: the maximum specific growth rate *(μmax)* and doubling time of different strains were highly correlated (Pearson correlation, r = -0.96, *p-value* < 0.0001). C) Maximum specific growth rate (μmax) variation among different strains. D) The growth of competing *Z.tritici* strains involved in different pairwise interactions can either be significantly or not significantly different from each other. Some strains can always either be the fast or slow-growing strain in all their significant interactions, whereas the situation is contrasted for some other strains as they can grow either faster or slower depending on the pairwise interaction.

Divergent outcomes of in-vitro pairwise interactions

We investigated the overall growth in mixed cultures compared to the growth of individual strains used to establish the co-culture. We found that 7 out of 91 co-cultures were growing not significantly different to one of the two strains to establish the mixture while the other strain grew significantly less alone (Fig. 2A; pattern *a*; for examples See Supplementary Figure S2). Furthermore, 17 out of 91 pairings showed the opposite pattern with one of the strains in the pairing growing significantly more

alone compared to the mixture (Fig. 2A; pattern *b*; for examples See Supplementary Figure S2). Additional patterns included either one or both strains growing alone significantly less than the co-culture (Fig. 2A; pattern *c* and *f*; for examples See Supplementary Figure S2). We also observed 7 co-cultures growing with no significant differences to both single strain cultures (Fig. 2A; pattern *d*; for examples See Supplementary Figure S2). Besides, we observed 7 co-cultures growing at an intermediate level with significant differences to both single strain cultures (Fig. 2A; pattern *g*; for examples See Supplementary Figure S2). Finally, we observed no significant difference in growth among either single strains or the co-culture for 38 out of 91 interactions (Fig. 2A; pattern *e*; for examples See Supplementary Figure S2).

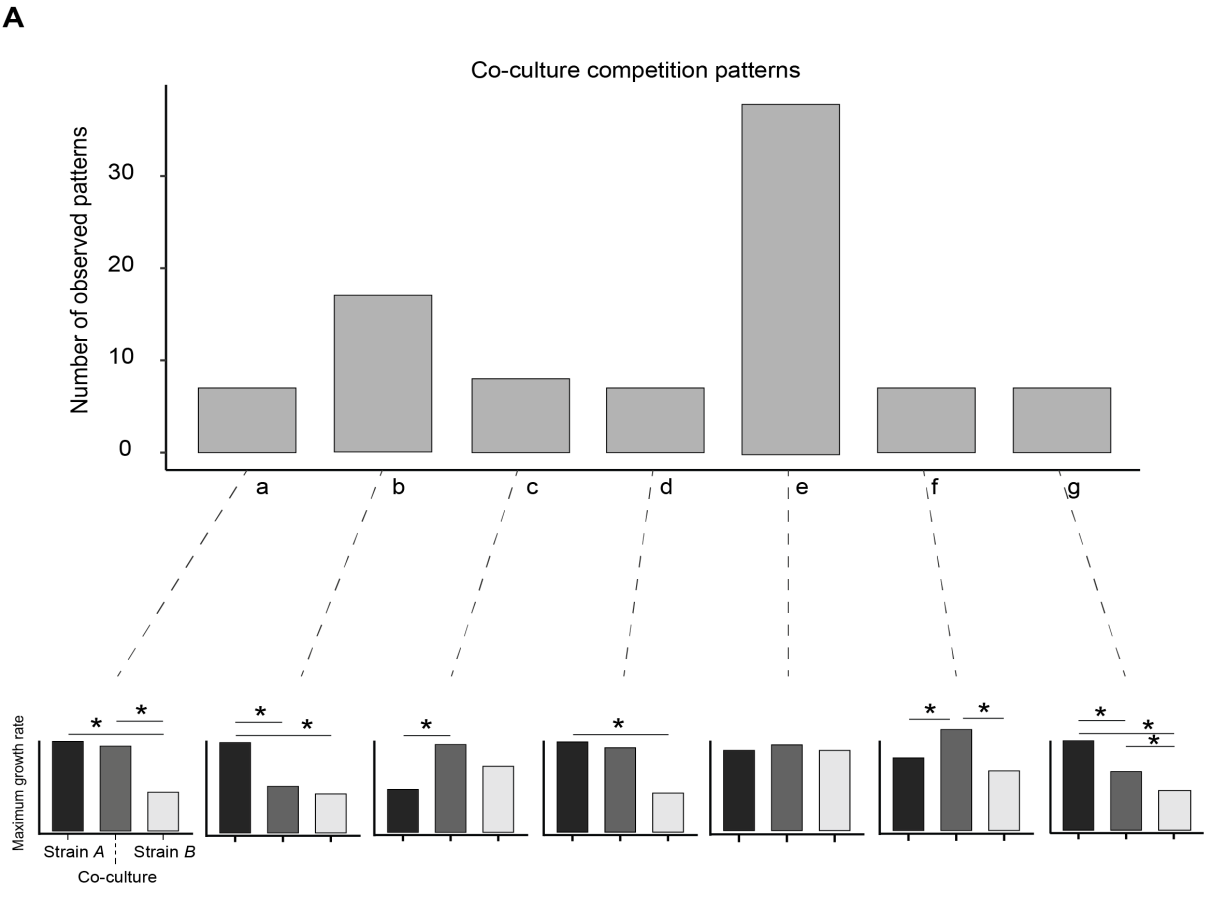

Figure 2: Divergent patterns of mixed culture outcomes during in-vitro experimental conditions. A). The count of pairwise interactions belonging to seven different outcomes patterns seen during in vitro mixed interactions. (The letters a, b, c, d, e, f and g indicating different patterns type).

Variation in infection outcomes among single and co-infections

We analyzed infection symptoms on wheat leaves produced both by single strain infections (n = 15) and 105 pairs created from the same set of strains. Disease symptoms were assessed as the percent leaf area covered by lesions (PLACL). The measurements were made using scans of entire wheat leaf and contrast-based image analysis (Fig. 3A; See Supplementary Table S4). Single strain infections produced variable disease symptoms from PLACL ranging from 0-70% (Fig. 3B). For the 105 different pairs, ten pairs were included strains with a significant difference in PLACL when infected alone (*p-value* < 0.05; Fig. 3C). The ten pairs included a total of ten different strains. We analyzed the outcomes of the co-infection compared to the single infections similarly to the culture conditions. We observed two and four out of 105 pairs with one strain being either significantly lower or higher, respectively, compared to the other strain and the co-infection (Fig. 4A-B; patterns *a* and *b*). We observed higher numbers of interactions where either one strain alone was producing significantly less symptoms compared to the co-infection or compared to the other strain (Fig. 4A-B; patterns *c* and *d*). Over two thirds (72 out of 105) of all interactions tests showed no significant differences between either strain alone or the co-infection (Fig. 4A-B; pattern *e*). Finally, the last relevant virulence pattern consisted of two strains were not statistically different, but the co-infection was significantly different from the 2 strains (*p-value* < *0.05*; Fig. 4A-B; patterns *f*). We found 7 out of 105 combinations tended to follow this pattern (Fig. 4A-B; patterns *f*). Interestingly in all tested combinations following this pattern, the virulence of co-infection was always lower than that observed during single infections.

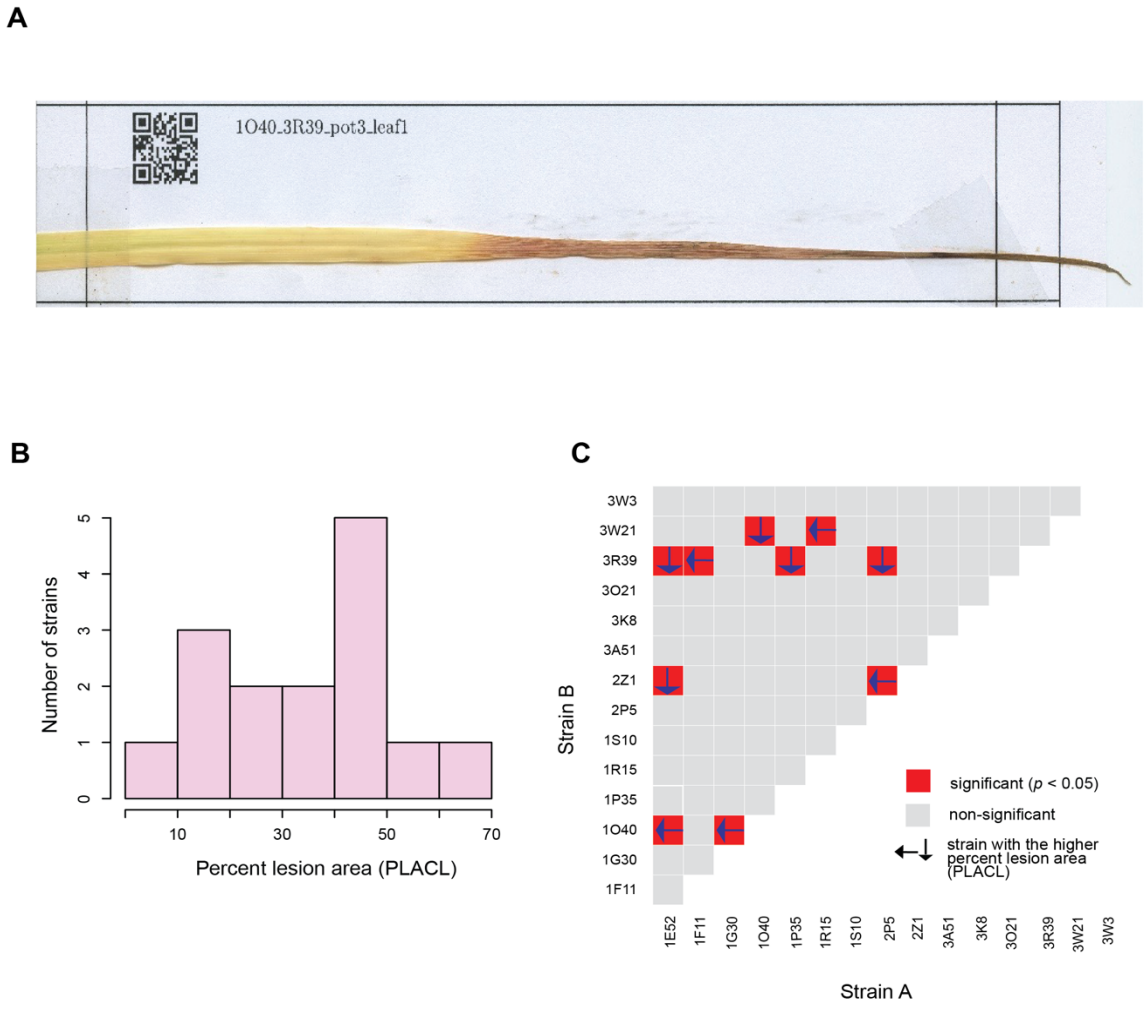

Figure 3: Virulence distribution among different *Z. tritici* strains. A) Wheat leaves were single or co-infected with 15 no clones *Z. tritici* strains during 5 different periods of infections. PLACL were then calculated for each single and co-infection respectively. B) Number of strains for each PLACL range. C) Variable virulence ability among different *Z. tritici* strains involved in different in planta pairwise interactions.

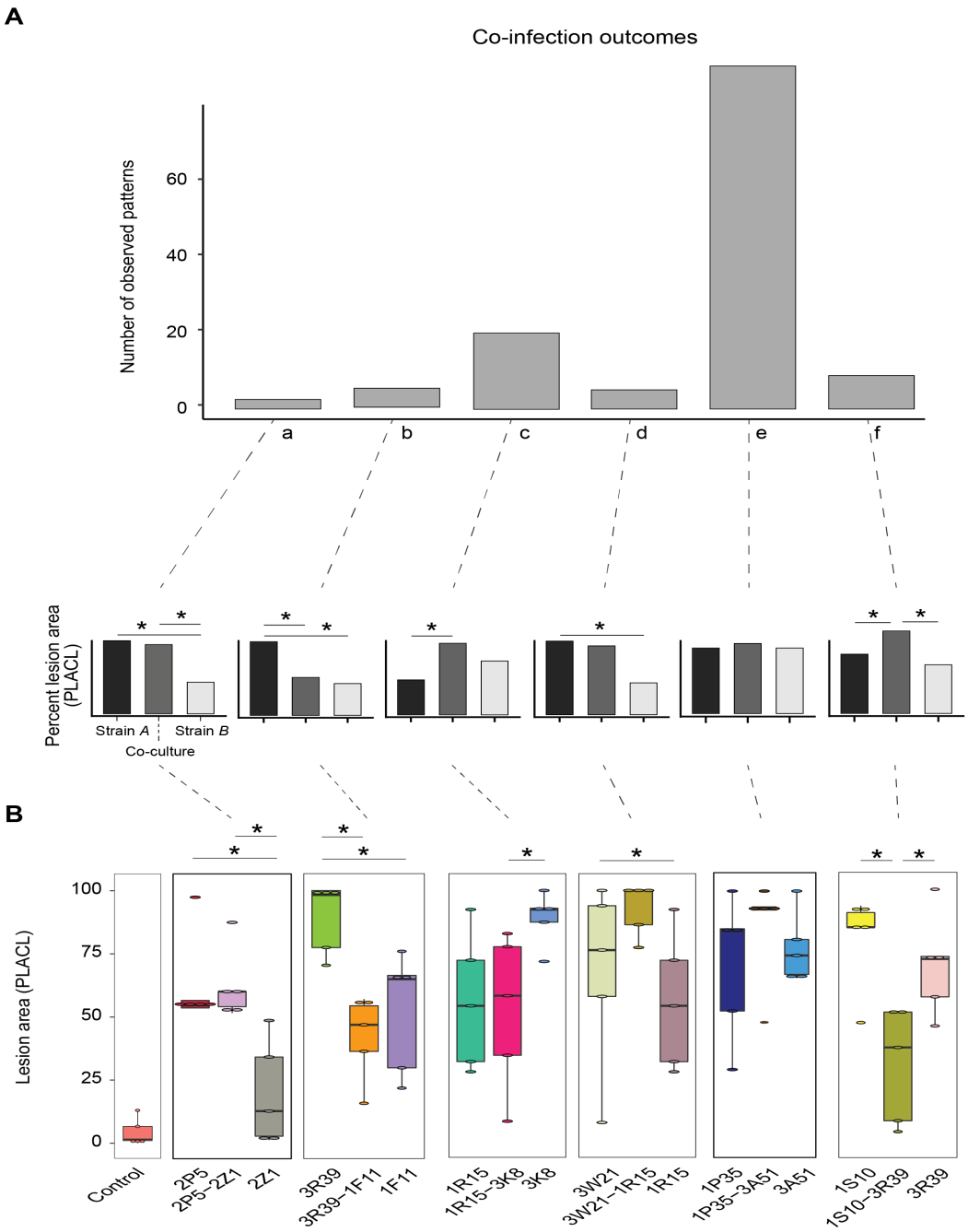

Figure 4: Divergent patterns of co-infection outcomes during in-planta experimental conditions. A) The count of pairwise interactions belonging to six different outcomes patterns seen during in planta mixed interactions. (The letters a, b, c, d, e, f indicating different patterns type). B) Some examples of in planta pairwise interactions belonging to different co-infection outcomes patterns.

Contrasting co-culture effects in vitro and during host infections

Given the differences in the environments in which we assessed co-culture and co-infection outcomes, we evaluated the similarity in outcomes of identical strain pairings for a total of 91 matched strain pairings. Single strains showed a moderate but significant correlation between maximum growth rate in culture and the lesion development on wheat leaves ($r = 0.55$, $p = 0.04$; Fig. 5A). Next, we evaluated whether the differences observed between individual strains and the strain mixture were consistent between culture medium and the wheat infection. We found that among all evaluated pairs 30 were found to produce matching interaction pattern and 61 were not matching between the environments (Fig. 5B; for examples See Supplementary Figure S3-4). As an example, the strain 3K8 was in single culture among the fastest growing strains, however 3K8 was not producing high amounts of lesions on leaves. In strain mixtures with 3K8, *in vitro* assays showed reduced growth compared to 3K8, while infection assays showed mostly more lesion development compared to the strain alone (Fig. 5C). The interaction between strains 1O40 and 1G30 was similar across conditions with 1O40 being a fast grower *in vitro* and producing high degrees of lesions in single strain assays. The mixture of 1O40 with 1G30 led to both weak growth in culture and less lesions on leaves (Fig. 5C). Finally, we found a majority of mixture assays with divergent outcomes between *in vitro* co-culture and plant co-infection (Fig. 5C). The mixture 1O40-1F11 produced higher growth than any individual strain, however no difference in lesion development was observed among strains and co-infection. In contrast, the mixture 2P5-1G30 showed no significant difference among any single or mixed culture. The same mixture produced more lesions on leaves than any single strain.

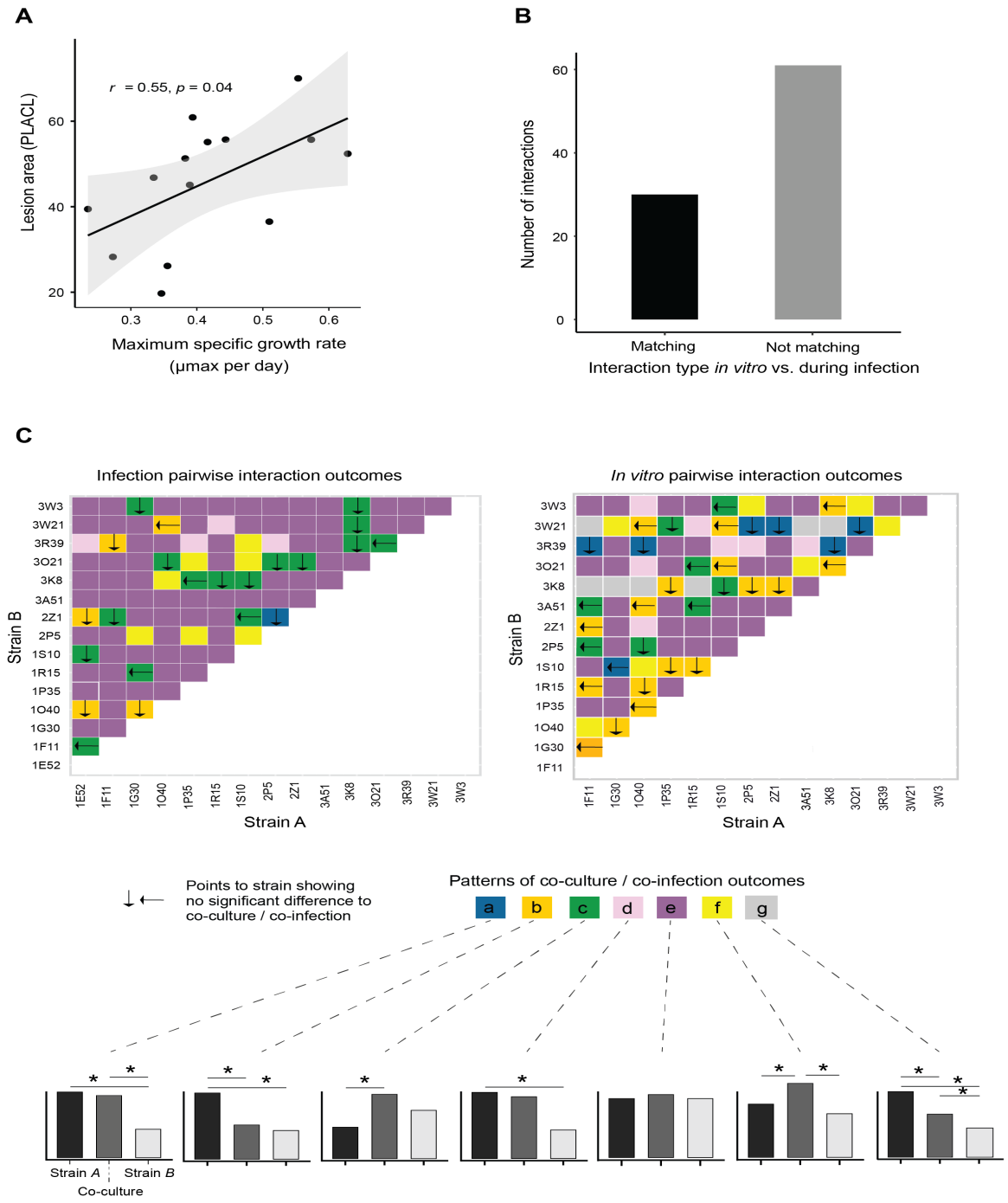

Figure 5: Comparison of in vitro vs. in planta pairwise interactions outcomes. A) Correlation between strain's growth rate and virulence outside and within plant host. B) Number of matching and no matching pairwise interactions outcomes outside and within a plant host. C) Divergent outcomes patterns of in-vitro and in planta mixed interactions. The letters a, b, c, d, e, f and g indicating different patterns type).

Discussion

Intraspecific co-infections are frequent in nature with plant hosts being often colonized by diverse strain sets (Susi et al. 2015; Walkowiak et al. 2015; Barrett et al. 2021). Yet, consequences of co-infections are often poorly understood. Our study shows that conspecific genotype pairings can produce divergent outcomes whether the interaction is assessed in culture conditions or as an infection phenotype. Moreover, highly virulent strains are not necessarily the most competitive outside of the host creating the potential for complex selection regimes over the life cycle of the pathogen.

Competition can be defined as the interaction occurring when organisms accessing a limited resource and having an impact on fitness (Lang and Benbow 2013). We found that during monoculture experiments outside of the host, individual strains showed genetic differences in their ability to grow on nutrient rich medium consistent with previous studies on *Z. tritici* populations (Lendenmann et al. 2016; Dutta et al. 2021). Controlling for the amount of inoculum, co-cultured strains varied widely in their joint growth rates. A large majority of co-cultures produced growth patterns consistent with neutral interactions (*i.e.* no competition). These cases included outcomes where neither the mono- nor the co-culture showed significant growth differences. Additional cases consistent with this scenario were patterns where the co-culture was showing an intermediate growth with either significant or non-significant differences to each monoculture. For strain pairs showing significant differences in monoculture growth, the co-culture was often growing similarly to the weaker strain. However, our experiments lacked the statistical power to clearly distinguish growth differences for most pairings. Despite the large number of tested pairs, we observed no co-culture with significantly reduced growth compared to both monocultures. Hence, strongly suppressive effects among strains appear to be rare under the tested conditions. Interestingly, a minority of pairings (~5%) showed enhanced growth in the co-culture compared to each monoculture. It is unknown how such growth acceleration may be triggered by sensing the presence of competitors. An important complement to test hypotheses on mechanisms involved in the competition will be to assess frequencies of individual strains in co-cultures by means *e.g.* of qPCR or fluorescent labelling techniques. A separate study of four fluorescence-tagged

Z. tritici strains showed a consistent pattern of fast-growing isolates also growing well in co-culture (Bernasconi et al. 2022). How fungi recognize self from non-self at different morphological stages and environmental conditions remains poorly understood though. Well investigated recognition systems include vegetative incompatibility reactions at the hyphal stage upon non-self-contact in *Neurospora crassa* and other filamentous fungi (Hartl et al. 1975; Aanen et al. 2010). Non-self-recognition may be important if strains were to modulate growth investment depending on conspecific competitors.

Strains of *Z. tritici* produce a variety of symptoms on wheat leaves during infection. We focused on lesion development after a successful infection by the pathogen. The observed range of lesion area per leaf for single isolate infections shows that all strains are capable of causing defense responses on the plant host. We observed no meaningful amount of asexual spore structures (*i.e.* pycnidia) in contrast to previous greenhouse experiments with the same pathogen and under field conditions (Singh, Karisto, et al. 2021). The lack of pycnidia production is most likely related to insufficient inoculation time, host resistance or environmental conditions not conducive for full infections. Nevertheless, the extent of lesion development on leaves is correlated with the success of the pathogen to colonize the leaf surface and trigger defenses. Under field conditions lesion and pycnidia development are largely correlated (Mikaberidze and McDonald 2020). Most strain pairs used for co-infections showed no significant differences in lesion development either as a single infection or co-infection. A considerable minority of strain pairs produced intermediate lesion development during co-infection compared to the two strains in single infections. This suggests that under most circumstances co-infections produce only weak deviations in infection outcomes compared to single strain expectations. However, we also found a small set of co-infection pairings where lesion development was accelerated showing that rare co-infections can increase the damage to the plant even at identical inoculum density.

Strain pairs producing higher growth compared to single strain growth *in vitro* did not match the strain pairs producing more lesions during co-infection. This mismatch is supported more broadly by having approximately two thirds of all tested co-infection pairs show divergent *in vitro* and plant infection outcomes. Hence, competitive outcomes *in vitro* are only weak predictors for strain interactions on the

plant. Such a mismatch between *in vitro* and infection outcomes was also previously observed in an investigation of four *Z. tritici* strains (Barrett et al. 2021). Interestingly, pycnidia production (a proxy for fitness) was generally reduced during co-infection compared to single strain infections (Barrett et al. 2021). This points to a potential cost of competition at the level of reproduction. However, larger numbers of strain pairings need to be analyzed under conditions allowing for pycnidia observations. Lesion development may only be weakly associated with reproductive output as lesions are largely an expression of host immune responses and damage through reduced photosynthesis. It remains to be investigated how increased lesion development is triggered by the presence of more than one genotype. During the infection process, *Z. tritici* produces hyphae that enter the intercellular space of wheat cells to produce pycnidia in the substomatal space (Kema et al. 2000; Palmer and Skinner 2002). Fluorescent microscopy analyses of co-infections showed that both hyphae and pycnidia are densely packed without apparent exclusion zones among genotypes (Bernasconi et al. 2022). Increased damage to the host during co-infection could be due to an accelerated hyphal growth of individual strains. Alternatively, different strains may produce different effectors and metabolites during infection, which could in combination cause greater damage.

The ubiquity of exposure to conspecific strains could create sufficient selection pressure to favor genotypes retaining growth or infection proficiency despite the presence of competing strains. We identified a small set of strains (n=4) retaining growth in each of their pairings with other strains, as well as two strains producing similar amounts of lesions even when exposed to other strains. However, genotyping of co-cultures and co-infections would be necessary to assess how well individual strains reproduce when exposed to other strains. Investigating strategies of pathogens coping frequently with high density environments and exposure to conspecific strains should be an important goal of future co-infection studies. Because co-infections are common in nature (Cox 2001; Read and Taylor 2001; Abdullah et al. 2017), understanding the evolution of virulence under co-infection is important for disease management and prevention. Mismatches of competitive outcomes between environment on and outside the hosts make predicting co-infection damage more challenging. However, systematic

screens of pairwise interactions combined with genetic analyses will help build more informed models of pathogen evolution on crops and other plants.

Methods

Pathogen strain selection

We used 14 and 15 genetically distinct strains of *Z. tritici* for *in planta* and culture condition experiments, respectively (See Supplementary Table S1). All strains were collected from the Field Phenotyping Platform (FIP) site of the ETH Zürich, Switzerland (Eschikon, coordinates 47.449°N, 8.682°E) in 2016 from 8 winter wheat cultivars (Karisto et al. 2018). After sampling, spores of each strain were stored in either 50% glycerol or anhydrous silica gel at − 80 °C (Singh, Karisto, et al. 2021). Illumina sequencing datasets are available for all used strains (Singh, Karisto, et al. 2021).

In vitro co-cultivation of conspecific strains

Z. tritici strains were revived in solid culture media Yeast-Malt-Agar (YMA) added with 50 µg/mL of kanamycin and incubated for 8-10 days in 18°C. A single colony of each strain was selected and inoculated in a 50 ml conical flask containing 20 mL liquid yeast sucrose broth (YSB) medium supplemented with 50 µg/mL of kanamycin. The inoculated flasks were incubated in the dark at 18° C and 140-180 rpm on an orbitary shaker for 8 days. After 8 days of incubation, the cultures were passed through four layers of sterile meshed cheesecloth (Oekostar Textile AG, Switzerland) to eliminate hyphal biomass and retain only spores. Spore suspensions were centrifuged for 15 minutes at 3700 rpm (Vaudaux-Eppendorf AG, Switzerland). After discarding the supernatant, the pellet was washed with sterile water to remove media traces. The process was repeated one more time. Retained spores were grown in 50 mL conical flask, each containing 20 mL of Vogel's minimal medium supplemented with 50 µg/mL of kanamycin. The flasks were inoculated with spores to reach a spore density of approximately 10^7 spores per mL. The cultures were then incubated in the dark at 18° C and 140-180 rpm. For growth rate monitoring experiments, spores were filtered, diluted in fresh minimal media, and the cell density adjusted to 10^5 spores/mL by counting the cells in a KOVA cell chamber system (Kova International, USA). Strains were then cultured alone or in pairs in 96-well plate in either 100 µL or

200 µL, respectively. The plates were sealed and incubated at 18 °C at 200 rpm on a shaker incubator for 11 days with optical density measurements (OD_{600}) once daily on a SpectraMax i3x plate reader (Molecular Devices, USA) with a shaking period of 5 s prior to the measurement. The OD was exported to Microsoft Excel for further analysis and generation of the growth curves. At least 5 replicates for each single or paired culture were set up (See Supplementary Table S3).

Wheat cultivar selection and infection assays

The wheat (*Triticum aestivum L.)* cultivar Combin was used due to its susceptibility to *Z. tritici (Schaad et al. 2019).* Five seeds of the cultivar Combin were placed in pots filled with compost soil and placed in white watering boxes. The pots were set to replicate (n=5) each treatment of single and co-infection. Wheat was cultivated during 21 days in a growth chamber with a photoperiod of 16h light / 8h dark, a temperature of 18°C and a relative humidity of at least 60%. Plants were watered when needed and randomized regularly. Strains of *Z. tritici* used in the infection experiment were revived from stock in solid culture media yeast malt agar (YMA) supplemented with 50 µg/mL of kanamycin as described above. Strains were cultured at a temperature of 18° for 8-10 days. Following this time period, a single colony of each strain was selected and used to inoculate 20 mL of liquid culture media containing yeast sucrose broth (YSB) supplemented with 50 µg/mL of kanamycin. The incubation was at 18°C on a shaker for 8-10 days at 160 rpm. Following this step, strains were filtrated using sterile cheese cloth (Oekostar Textile AG, Switzerland) and placed in a cold room at 4°C. Then, 3-5 days before their use in the experiment, the strains were taken from the cold room and 500µL of each strain was used to inoculate 20 mL of liquid YSB and placed in a shaker at 140-160 rpm and 18°C. The strain cultures were filtered using sterile cheese cloth (Oekostar Textile AG, Switzerland) and centrifuged for 15 min at 3700 rpm (Vaudaux-Eppendorf AG, Switzerland). After discarding the supernatant, the pellet was resuspended in 20 mL of Milli-Q water (Merck Group, Switzerland) and centrifuged again for 15 min at 3700 rpm. This step was repeated once more. The spore concentration of each strain was adjusted to either 10^5 spores per mL or half the concentration for co-infections using a hemocytometer (Kova International, USA). The strains were diluted in Milli-Q water (Merck Group, Switzerland) to reach the desired concentration of 15 mL. Finally, 0.1% of Tween 20 (Sigma-Aldrich, Switzerland) was added

to facilitate wheat leaf spore adhesion. Treatments consisted of plants sprayed with water only (control treatment), plants infected with one strain at 10^5 spores per mL (single strain control), or plants co-infected with two strains with a concentration of 5 * 10^4 spores per mL per strain (co-infection treatment). After, twenty-one days of seed sowing, each pot was sprayed separately protected by cardboard to prevent splashing. Sprayers containing either spore suspensions or sterile Milli-Q water (Merck Group, Switzerland) were used. Each pot was placed in white container boxes and sealed with plastic bags and adhesive tap after being watered. The sealing helps maintaining 100% humidity in a room at 18°C for 48 hours. After this period, each plastic bag was opened and left in the climate-controlled room for 21 days. The plants were watered when needed and pots were regularly randomized within each white box. White boxes were also regularly randomized.

Plant infection assessments

After twenty-one days of infection, the first two leaves of each plant were cut and pasted on paper sheets containing a QR code for tracking. Then, each sheet was scanned at 1200 dpi using a flatbed scanner (EPSON perfection V55O). Following this step, an automated image analysis was performed using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018). We used a macro developed to assess *Z. tritici* disease symptoms (Karisto et al. 2018) to determine the percentage of leaf area covered by lesions (PLACL), which is a proxy for leaf damage and virulence (Stewart and McDonald 2014). Automatic assessments were visually checked for consistency. PLACL values were adjusted if color thresholds were not appropriately distinguishing yellowish (*i.e.,* lesion areas) and green (*i.e.,* healthy) areas on some leaves. Overall, very little pycnidia formation was observed during the infection period and pycnidia counts were, therefore, not considered further.

Data analyses

All data analyses were carried out using R v4.0.4 software (R Core Team 2021b). Optical densities were corrected for the blank control (Vogel's minimal media) by subtracting the mean optical density for each strain. Fungal growth curves were estimated by fitting a log-logistic model to the data using

the {Growthcurver} R package (Sprouffske and Wagner 2016). Furthermore, {Growthcurver} was used to summarize the following growth characteristics: generation time (t_d) and the maximal specific growth rate (μ_{max}). Visualizations were made using the {ggplot2} R package (Wickham 2016). Analysis of single versus co-culture data was performed using one-way ANOVA and Tukey-Kramer tests for post-hoc analysis in R. To satisfy normality assumptions, PLACL data was square-root arcsine transformed. Data handling was performed with the R packages {dplyr} (Wickham et al. 2022), {reshape2} (Wickham 2007) and {tidyr} (Hadley and Maximilian 2022). We used a linear model to test the effect of the different treatments on PLACL, followed by a one-way ANOVA with the function "Anova" from the {car} package (Fox and Weisberg 2019). Assumptions of linear models were visually assessed with the function "autoplot" from the package {multcomp} (Hothorn et al. 2008). Post-hoc analysis was done using the "emmeans" function from the package {emmeans} (Lenth et al. 2018) with the Tukey-Kramer method for multiple testing correction.

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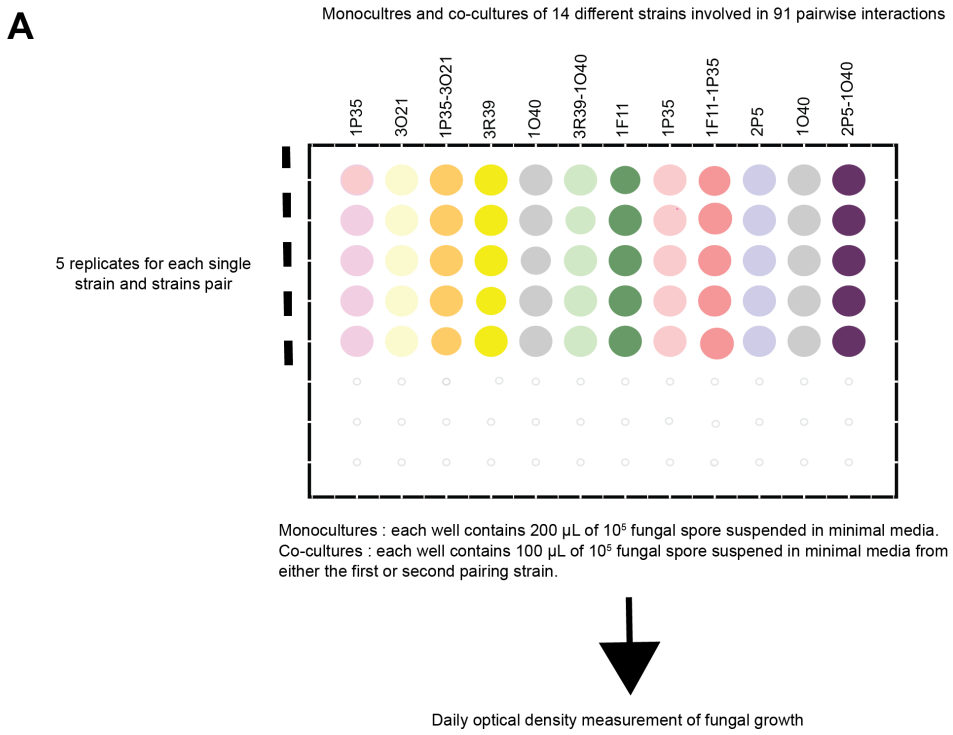
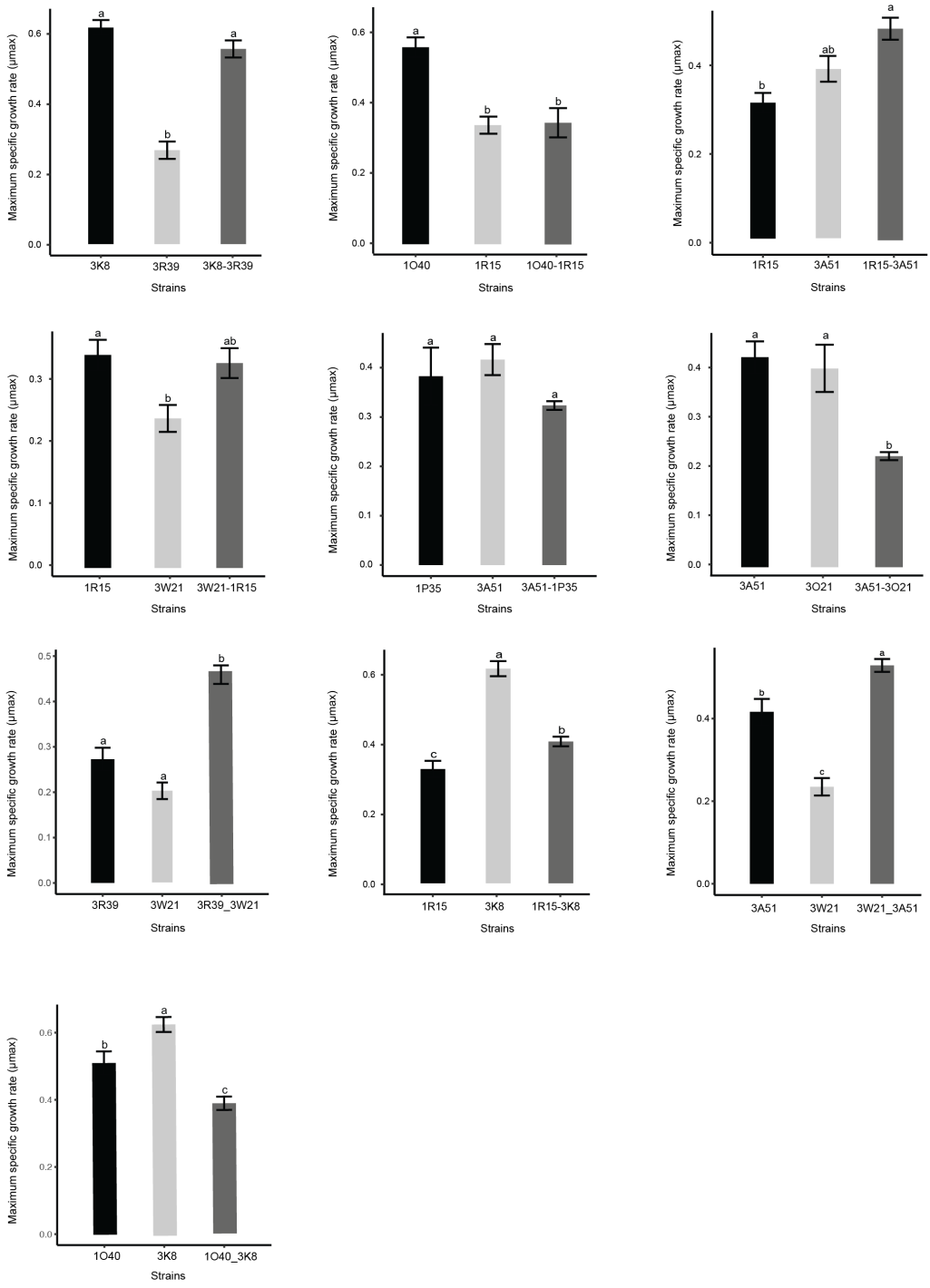
Supplementary Information

Supplementary Figures

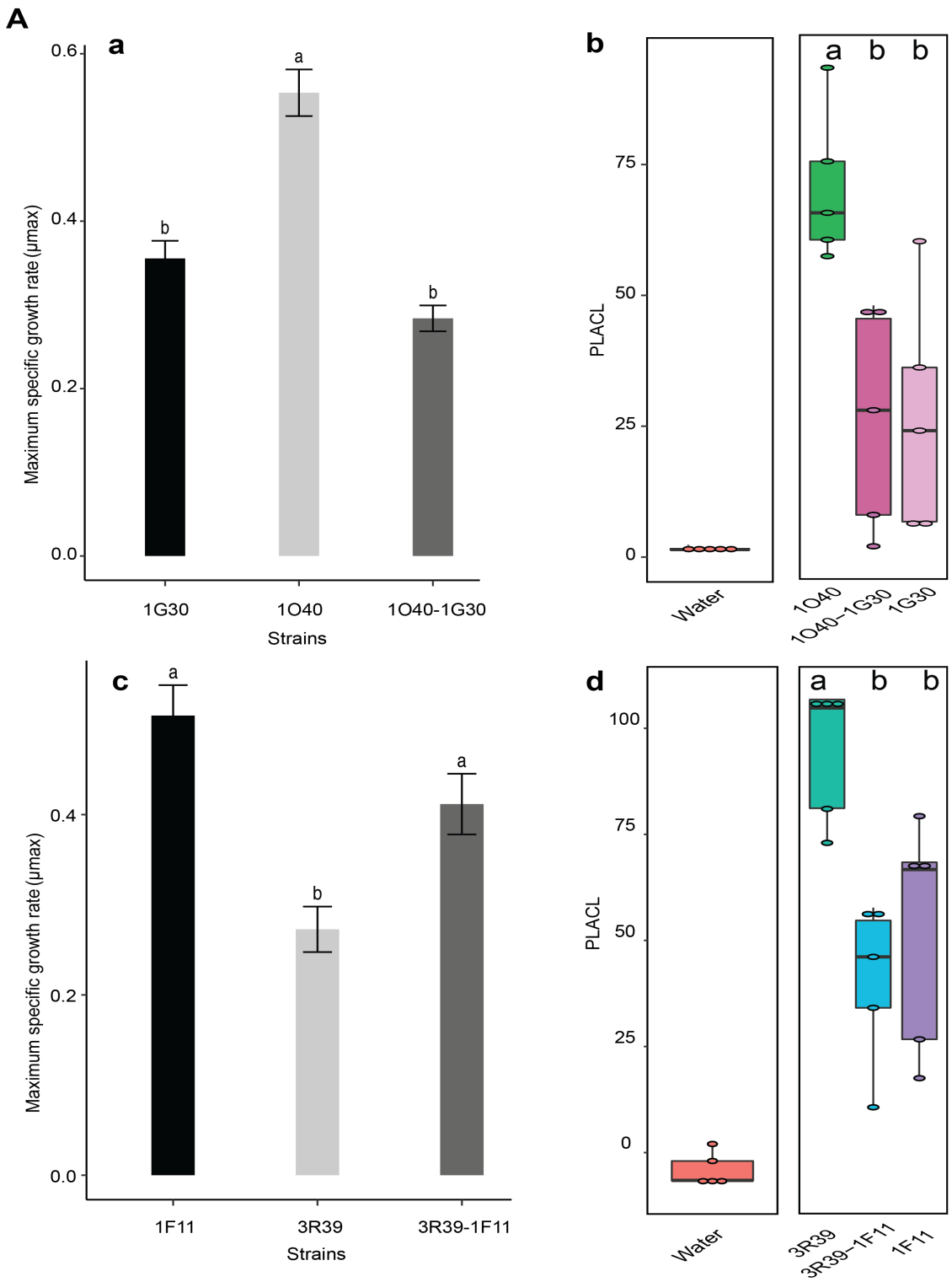


Supplementary Figure S1: In-vitro *Z. tritici* strain's culture growth metrics assessment. A) Schematic overview of in-vitro fungal strains growth assessment during single and mixed culture using optical density (OD) measurements. For each monoculture a total volume of 200 µL of fungal strain spore concentration of 10^5 suspended in minimal media was cultured in each microplate well, whereas for mixed culture, each microplate well was containing 100 µL of fungal strain spore concentration of 10^5 suspended in minimal media from either the first or second pairing strain. At least 5 replicates for each single or paired culture were set up. Microplates were then incubated in dark at 18°C and daily optical density (OD) was measured.

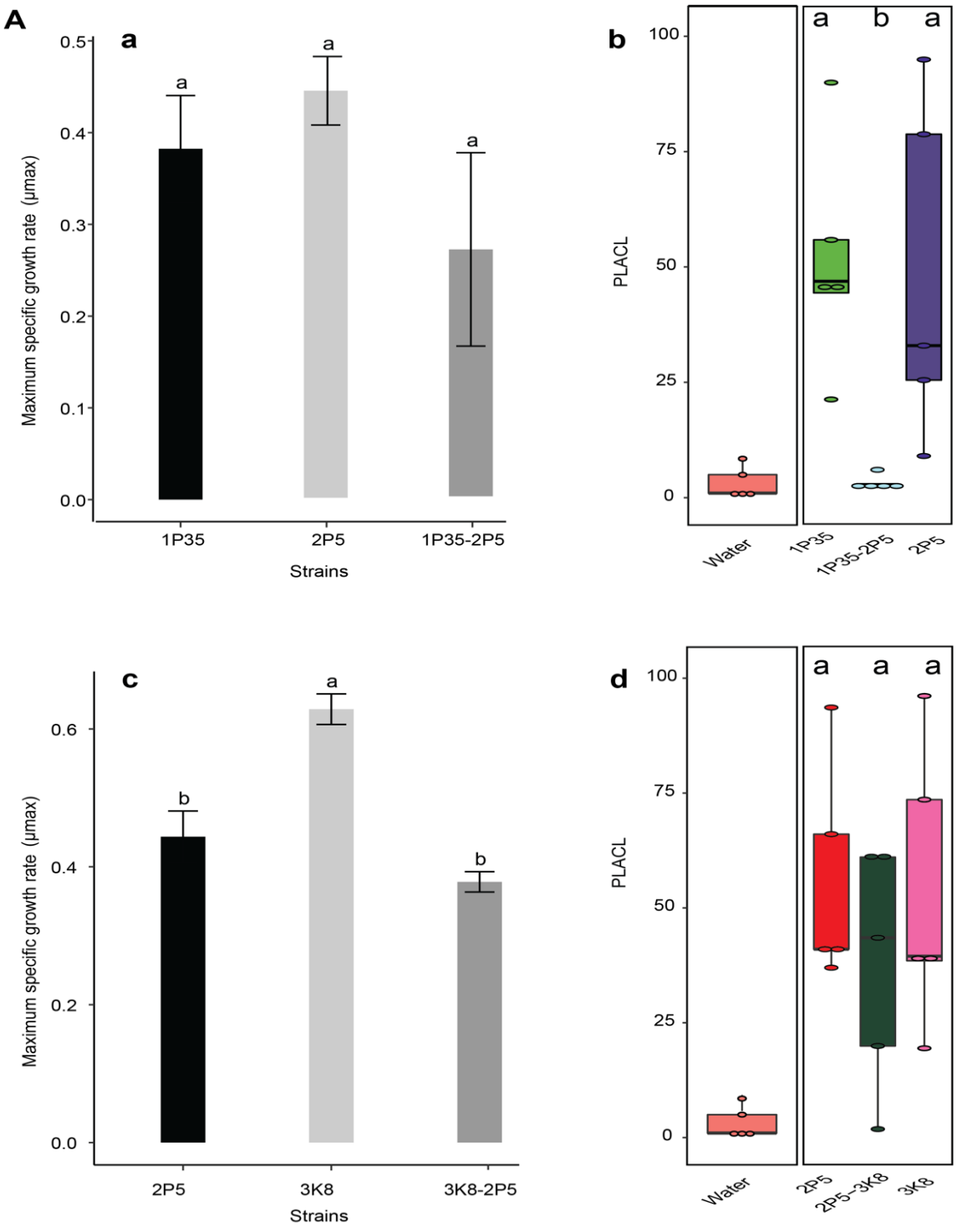
A



Supplementary Figure S2: *Z.tritici* strains examples for each in-vitro pairwise interaction outcome pattern.



Supplementary Figure S3: Examples of matching pairwise interactions outcomes outside and within plant host.



Supplementary Figure S4: Examples of no matching pairwise interactions outcomes outside and within plant host.

Supplementary Table legends

(See Excel file)

Supplementary Table S1: Strains used either in vitro or in planta pairwise interactions experiment.

Supplementary Table S2: Name of strain's pair combination tested either in-vitro or in planta pairwise interactions experiment.

Supplementary Table S3: Strain's optical density (OD) and growth metrics measurement.

Supplementary Table S4: PLACL measurement across infected wheat leaves by different strains.

Chapter 3

Diverse sets of transposable elements contribute to the rapid sequence evolution of virulence and fungicide resistance loci in the fungal wheat pathogen *Zymoseptoria tritici*

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Abstract

Rapid sequence changes and genetic diversity drive the evolution of virulence and drug resistance in microbial species. In fungal plant pathogens, the insertion of transposable elements (TEs) is considered to be a key source of genetic variation. However, the dynamics of TEs insertions associated with virulence and fungicide resistance genes remain poorly understood at the species level. To investigate TEs insertion dynamics in *Zymoseptoria tritici*, a fungal pathogen of wheat, we used a dataset of 1012 isolates with available draft genomes covering populations across six continents. We identified a considerable number of distinct TEs insertions from different TE families nearby two genes encoding major effectors involved in plant infections. Insertions were rare nearby two fungicide resistance loci with geographic patterns consistent with the rise of fungicide resistance. We also found singleton insertions indicating ongoing activity TE generating additional genetic diversity. Our study provides a large-scale repertoire of intra-species genetic variation in TEs near genes underlying recent adaptive evolution. The extent and diversity of TE insertions highlights the importance of selfish element activity for pathogen evolution.

Introduction

Transposable elements (TEs) are repetitive mobile genetic elements first discovered in maize (McClintock 1950). In eukaryotic genomes, the repeat content can differ between species with 46% in the human genome (Lander et al. 2001), up to 85% in maize (Vicient 2010). In fungi, genome show a wide range of TE content with up to 90% for the powdery mildew *Blumeria graminis* (Frantzeskakis et al. 2018). There are two major categories of TEs based on their replication mechanism, namely class I TEs, that transpose using an RNA intermediate (i.e., RNA transposons) and class II TEs, that transpose using a cut-and-paste mechanism (i.e., DNA transposons) (Wicker et al. 2007). Additionally, TE classes can be subdivided into a variety of families and superfamilies based on their coding sequence structure (Wicker et al. 2007). Some TEs are autonomous because they encode the necessary machinery to execute their own transposition (e.g., long terminal repeats, LTR). Several TEs, however, lack genes necessary for mobility and are called non-autonomous TEs (e.g., MITEs). Such TEs rely on proteins encoded by other ('autonomous') TEs (Wicker et al. 2007). Through their replication and insertion activity, TEs can create genetic diversity within species. While most TE insertions have no or detrimental consequences for the fitness of the host, numerous species have gained important adaptation to their environment through the action of TEs (McCullers and Steiniger 2017; Akakpo et al. 2020; Domínguez et al. 2020; Luo et al. 2020).

Fungal plant pathogens are exposed to a variety of environmental pressures including changing climatic conditions, various fungicide and host immune responses during infection (Croll and McDonald 2017; Li, Lu, et al. 2020). Driven by large population sizes and short generation times, pathogens can adapt rapidly to environmental challenges. Adaptive mutations conferring beneficial changes in virulence, fungicide resistance or stress tolerance were often found to be associated with the dynamics of TEs in the genome (Omrane et al. 2017; Krishnan et al. 2018; Porquier et al. 2021). Hence, TE insertions are key drivers for generating relevant genetic variation in populations and impact the evolutionary trajectory of pathogen species. During plant infection, pathogens secrete proteins called effectors, which target and inhibit plant defense mechanisms. Effectors are enhancing pathogen virulence and facilitating

infection (De Wit et al. 2009). In turn, the plant host uses immune receptors to recognize and defend against pathogen colonization (De Jonge et al. 2012; Selin et al. 2016; Jones et al. 2019; Tariqjaveed et al. 2021; Lazar et al. 2022). It is thought that effector proteins that evade detection or block defense signaling coevolve rapidly with their plant targets, that are playing role in detecting pathogens and trigger immune defenses (Fouché et al. 2018; Sánchez-Vallet et al. 2018; Han 2019; Badet and Croll 2020; Białas et al. 2021). TE insertions provide an important source of effector gene diversification facilitated by physical proximity of effector genes and TE-rich genomic compartments in diverse pathogens (Grandaubert et al. 2014; Soyer et al. 2015; Torres et al. 2020). TE activity can directly be underpin pathogenicity and suppression of plant defense-related genes during infection (Hartmann et al. 2017; Porquier et al. 2021). Moreover, genetic variation generated by TE insertions was shown to play also an important role in the evolution of fungicide resistance (Chen et al. 2017; Omrane et al. 2017). Overall, TEs contribute broadly to adaptive phenotypic variation relevant for pathogen adaptation.

The impact of individual TEs insertions in fungal genomes can range from disrupting protein-coding regions, altering gene expression, or inducing large chromosomal rearrangements (Krishnan et al. 2018; Torres et al. 2021; Demené et al. 2022). Purifying selection can efficiently eliminate deleterious TEs in at least some species (Oggenfuss et al. 2021). Beyond counter-selection, fungi have evolved mechanisms to suppress harmful effects of TE activity including DNA methylation (Montanini et al. 2014; Deniz et al. 2019), histone modifications (Soyer et al. 2021) and RNA-mediated silencing (Laurie et al. 2012; Lax et al. 2020). A fungal-specific genome defense mechanism to prevent TEs expression called repeat-induced point mutation (RIP) has first been shown in *Neurospora crassa* and more recently studied in a broad range of plant pathogens (Ikeda et al. 2002; Rouxel et al. 2011; Van Wyk et al. 2019). RIP is a mechanism targeting repetitive genomic regions by selectively mutating duplicated sequences and inducing single nucleotide mutations from C:G base pairs to T:A (Selker 1990). The genomes of many fungal pathogens have a high density of TEs and signatures of RIP activity (Grandaubert et al. 2014; Fokkens et al. 2018). Interestingly, the RIP mechanism can lead to virulence gains through rapid effector gene diversification (Van de Wouw et al. 2010; Singh, Badet, et al. 2021).

Hence, both beneficial TEs insertions and RIP activity can accelerate the evolution of virulence. Investigating TEs near effector genes within species and at population scale can provide important insights into TE-mediated effector evolution over short time scales.

The fungal pathogen *Zymoseptoria tritici* causes one of the economically most important wheat diseases called Septoria tritici blotch (STB) (Fones and Gurr 2015). The genome harbors 13 core chromosomes and up to 8 accessory chromosomes, which are not found in all strains of the species (Goodwin et al. 2011). TEs content varies among genomes of different strains (Badet et al. 2020). Moreover, accessory chromosomes contain higher proportions of TEs than the core chromosomes (Croll and McDonald 2012; Grandaubert et al. 2015). TEs have contributed to pathogenicity evolution through several mechanisms (i); TE insertions contributed to the diversification of effector genes (Krishnan et al. 2018; Meile et al. 2018); (ii) causing gene gain or loss or (Hartmann et al. 2017); (iii) leading to large scale chromosomal rearrangements (Badet et al. 2020, 2021). Environmental stress caused by plant infections has been found to de-repress the transposition activity of TEs during infection (Fouché et al. 2019). Moreover, multidrug fungicide resistance in field strains originated from the overexpression of the *MFS1* gene coding a multidrug transporter by the insertion of a TE in the promotor region (Omrane et al. 2017). A weakening of genomic defenses in the recent evolutionary history of the species has likely favored increased TE activity (Feurtey, Lorrain, et al. 2022). Moreover, TEs insertion triggered early stages of genome expansion among different populations (Oggenfuss et al. 2021).

Here, we investigated the presence of TEs near two major virulence and two fungicide resistance genes using a large draft genome assembly dataset of *Z. tritici*. To understand the dynamics of TEs, we first analyzed insertions dynamics in 1012 diverse isolates across six different continents and at the population level for two loci involved in fungicide resistance and two loci encoding major effectors. Next, we focused on a subset of rare TEs (*i.e.,* singletons) and analyzed the characteristics of the insertion sites. Our work provides a detailed map of major genes how TE insertion dynamics can generate potentially adaptive genetic variation.

Results

Detection of TE dynamics in virulence and fungicide resistance genes among populations

We analyzed 1012 *Z. tritici* isolates collected from 42 countries and six continents to investigate within-species TE dynamics. The genotypes of individual isolates were grouping into eleven genetic clusters retracing the global spread of the pathogen (Feurtey et al. 2022). We analyzed regions located within ~2 kb up- and downstream of the *AvrStb6* and *Avr3D1* genes encoding major effectors, as well as *CYP51* and *MFS1* genes both known to be associated with azole and multidrug resistance. We detected a total of 3408 TE fragments nearby the effector genes originating from 65 families TE (See Supplementary Table S1). For the *AvrStb6* gene, 2040 TEs were found and detected in 945 isolates (93.3%), whereas for the *Avr3D1* gene, 1368 TEs were detected in 1010 isolates (99.8%; Fig. 1A). The most widespread was from the Maki family in *Avr3D1* (n ~ 1004 isolates) and *AvrStb6* (n = 732), with each isolate carrying only a single locus Maki TE insertion. Additional TE families that were either single or multi-locus insertions were also found and showed variability among isolates (Fig. 1C-D and See Supplementary Figure1). For the two fungicide resistance loci, we identified a total of 571 TEs fragments from 57 different TEs families across both *CYP51* and *MFS1* genes (See Supplementary Table S1). For the *CYP5*1 gene, we found 427 TE insertions in 278 isolates (Fig. 1A). We also found that most of these isolates (n = 189) were carrying RNA retrotransposons belonging to the LARD_Gridr TE family (RLX superfamily; Fig. 1E). For the *MFS1* gene, 144 TEs insertions were identified across 125 isolates (Fig. 1A). A DNA transposon belonging to the Grace family and the DTM superfamily was found most frequently (41 isolates; Fig. 1F). We examined frequencies of TE families within each isolate. Interestingly, for some isolates, the frequent TE insertion from the LARD_Gridr TE family occurred in more than one locus in the *CYP51* gene for a total of 200 insertions of the LARD_Gridr across 189 isolates. For *MFS1*, the TEs insertions from Grace family were located in 41 different loci near the *MFS1* gene in 41 different isolates with each isolate carrying only a single Grace TE insertion (See Supplementary Figure1).

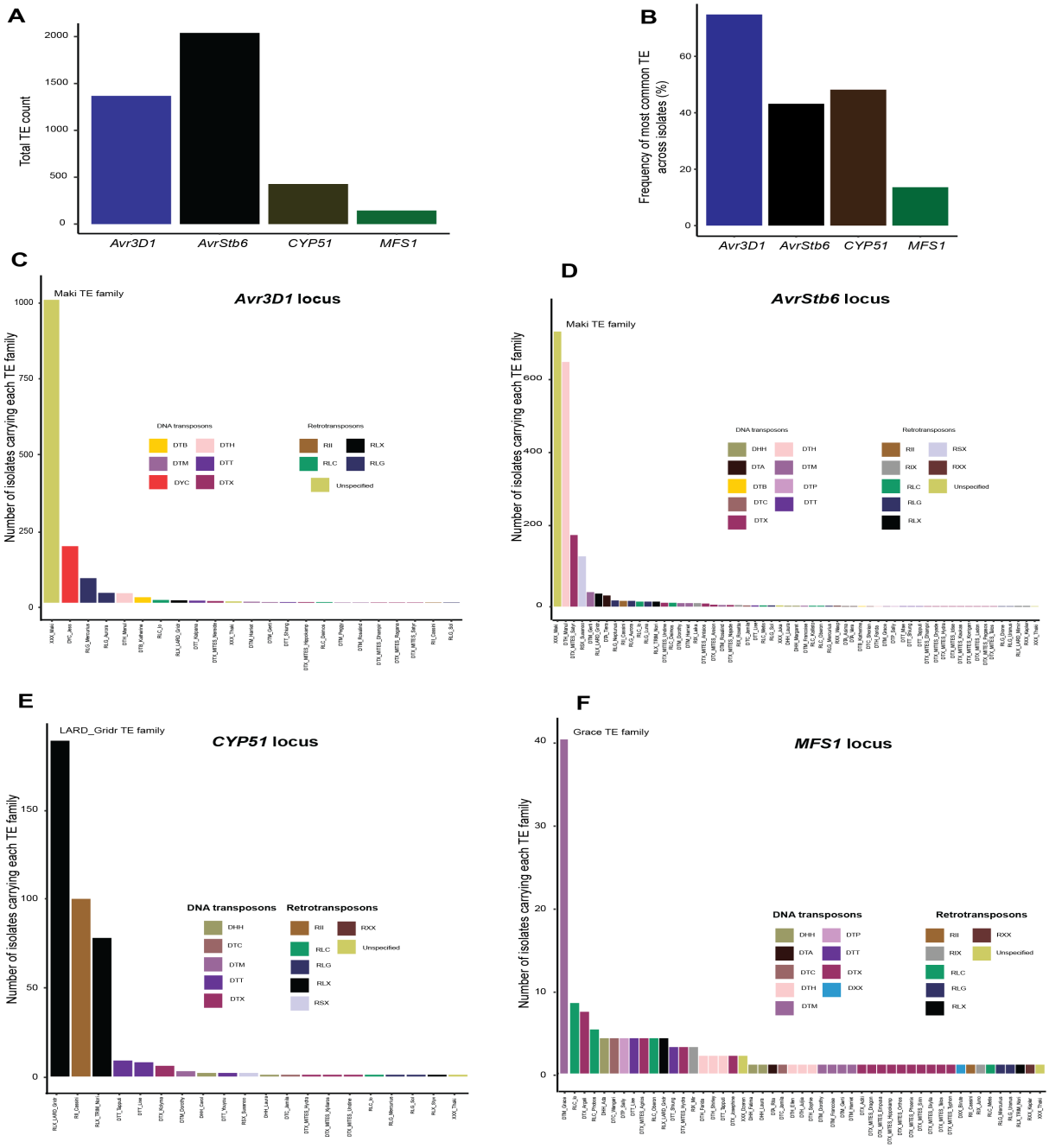

Figure 1: TEs count and most frequent TEs families in fungicide resistance and virulence loci. A) Total TE count per loci B) Frequency of most common TE in fungicide resistance and virulence genes loci across isolates C) The count of isolates carrying each TE family in *Avr3D1* locus. D) The count of isolates carrying each TE family in *AvStb6* locus. E) The count of isolates carrying each TE family in *CYP51* locus. F) The count of isolates carrying each TE family in *MFS1* locus.

Singleton TE insertions

We used visual inspection of multiple sequence alignments among the 1012 isolates to identify singleton TE insertions at the *Avr3D1*, *AvrStb6, CYP51* and *MFS1* loci. We assessed the reliability of a

singleton TE detection by analyzing homology of sequences up- and downstream of a TE insertion (See Supplementary Figures S2-3). Sequences belonging to a singleton TE insertion were then classified using Repeatmasker (See Supplementary Table S2). Among the 1020 isolates and the reference genome isolate IPO323, we identified a total of six singleton TE insertion at the *Avr3D1*, *AvrStb6, CYP51* and *MFS1* loci (See Supplementary Table S3). The singleton TE insertion in *CYP51* with conserved flanks belongs to the Carol TE family. The *MFS1* locus carries multiple singleton TE insertions belonging to the Cassini and Dzeren families. A the *AvrStb6* locus, we found two singletons from MITE Korrigan and Deimos families. The two detected TE singletons at the *Avr3D1* locus belong to the Ragana and Nereide MITEs families, respectively.

Differentiation in transposable element insertion frequencies within and among populations

To assess whether the TEs insertion dynamics reflected the global population structure, we analyzed the frequencies of the most frequent TE loci of all isolates (Fig. 1B). For *Avr3D1*, the most frequent TE was found in 74.85 % of all isolates from different populations and genetic clusters. We also found that the most frequent TE insertion (43.17%) at the *AvrStb6* locus was shared by almost half of the populations. The most frequent TE insertion at the *CYP51* locus was shared by 48.2 % of all isolates. Finally, for *MFS1*, the most common TE insertion was shared by 13.6% of all isolates. We found a strong geographic differentiation of the most frequent TE at both virulence and fungicide resistance gene loci. In the European genetic cluster, 300 out of 519 isolates (~57.80%) carried the most frequent TE belonging to Maki family at the *Avr3D1* (Fig. 2A). Similarly, the most frequent TE at the *AvrStb6* and *CYP51* loci from Maki and LARD_Gridr families respectively were at high frequency in the European genetic cluster (Fig. 2B and 3A). Overall, the most frequent TE at *Avr3D1* was geographically more widely spread compared to the major TE at *AvrStb6*. In contrast, the most frequent TE insertion in *CYP51* and *MFS1*, respectively, was abundant in only few genetic clusters showing considerable geographic differentiation (Fig. 3B). The frequent TE at the *MFS1* locus originating from Grace family was only found in Oceanian populations, while the frequent TE at the *CYP51* locus was only found in European and New Zealand populations.

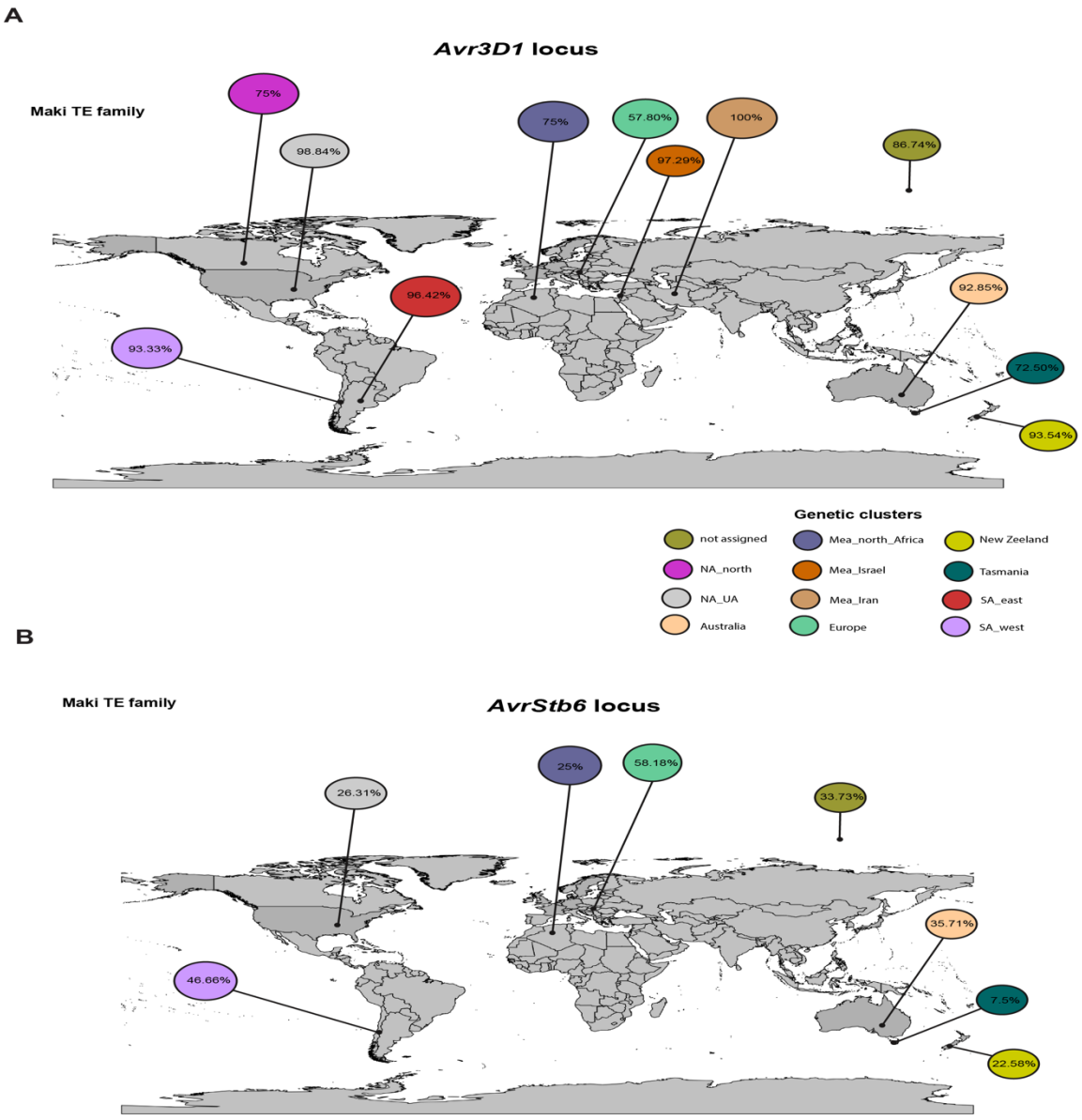

Figure 2: Geographic differentiation of the most frequent TE at virulence gene loci. A) Geographical distribution of the most frequent TE in *Avr3D1* locus. B) Geographical distribution of the most frequent TE in *AvStb6* locus.

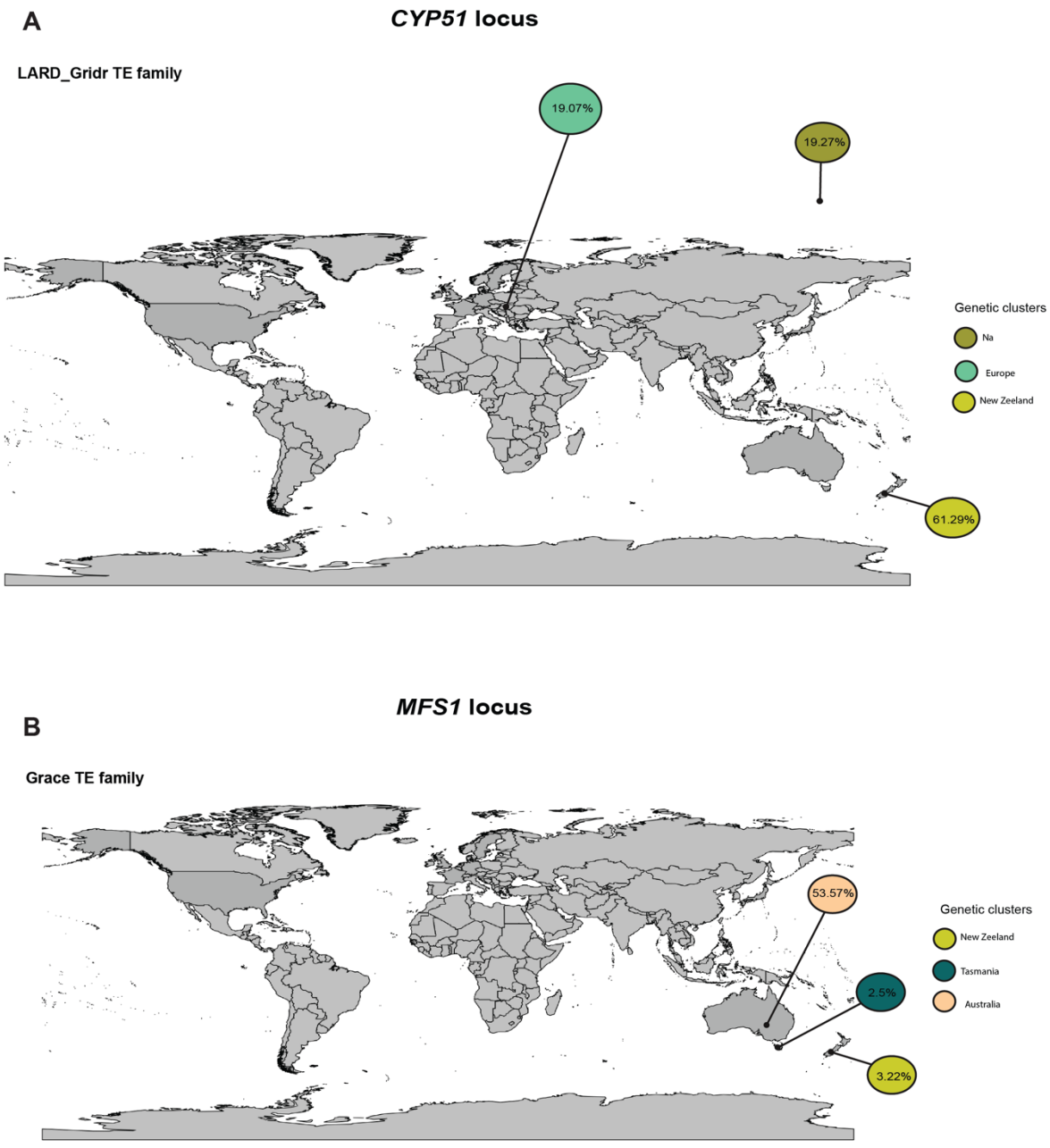

Figure 3: Geographic differentiation of the most frequent TE at fungicide resistance gene loci. A) Geographical distribution of the most frequent TE in *CYP51* locus. B) Geographical distribution of the most frequent TE in *MFS1* locus.

Discussion

TEs have played important roles in fungicide resistance and virulence evolution of *Z. tritici*. Here, we identified insertions of TEs from different TE families and superfamilies nearby major virulence and fungicide resistance genes. We also detected high-confidence singleton insertions most likely representing recent insertions. This study represents the first comprehensive study of TE insertions dynamics nearby major genes in *Z. tritici* using a global dataset.

We identified a high number and geographically structured TEs insertions at the *MFS1* and *CYP51* fungicide resistance loci. TEs insertions at these loci are known to mediate fungicide resistance in *Z.tritici* with a LTR retrotransposon insertions into the promoter region of *MFS1* causing overexpression and enhanced multidrug fungicide resistance (Omrane et al. 2017). LTR retrotransposon insertions nearby *CYP51* can also induce overexpression leading to enhanced azole fungicide resistance in the fungus *Blumeriella jaapii*, a cherry leaf pathogen (Ma et al. 2006). TE insertions also play a role in regulating melanin production in *Z.tritici* under stressful conditions by modulation the expression of a secondary metabolite gene cluster (Krishnan et al. 2018). However, little is known about the presence and distribution of TEs in pathogenicity-related genes. We found a considerable number of TE insertions from different TE families in *AvrStb6* and *Avr3D1* suggesting that TEs could play important roles in modulating virulence. We found considerably more TEs insertions in two analyzed virulence genes compared to the two fungicide resistance genes. Such differences might be due to various forces that shape TE insertion dynamics. Genetic drift can play a role in changing frequencies or interfering with the removal of deleterious TE insertions. Finally, the fitness gains through beneficial insertions play also an important role (Bourque et al. 2018; Stritt et al. 2018b). Individual TE families show also distinct activities across *Z. tritici* populations (Oggenfuss et al. 2021). The identity of TE families among virulence and fungicide resistance loci also varied widely. Maki represented the most prevalent TE family at both virulence loci. Interestingly, *CYP51* and *MFS1* loci were not sharing the same most common TE family suggesting distinct TE activity histories. TE families and superfamilies can have target site preference with *e.g.* some retrotransposons favoring insertions upstream of polymerase III

transcribed genes (Sultana et al. 2017). Some TE superfamilies preferentially insert into coding regions and transcription start sites (Gilly et al. 2014). Hence, the difference observed in the most frequent TE family could well be explained by insertion site preferences of the Maki family preferring genomic features predominantly found in the repeat-rich regions harboring *Avr3D1* and *AvrStb6*. Fungicide resistance genes tend to be embedded in more conserved regions possibly providing less opportunity for preferred TE insertions. However, overall lower TE numbers near fungicide resistance loci make direct comparisons of insertion preferences challenging and genome-wide investigations would be necessary.

Our analyses revealed multiple rare (*i.e.* singleton) TE insertions. The occurrence of loci with low-frequency TEs is consistent with recent genome-wide analyses of a subset of our analyzed populations (Oggenfuss et al. 2021). Low frequencies are consistent with purifying selection against TEs but also ongoing TE activity creating new insertions. Purifying selection against TE insertions is well established with strong signatures *e.g.* in *Drosophila melanogaster* (Luo et al. 2020). The global frequency analyses of TEs across the four loci suggested though that some high frequency TEs may be driven by positive selection and confer benefits. We found substantial TE frequency variation among genetic clusters and geographies, which may not be explained by genetic drift alone. Virulence genes can undergo rapid evolutionary change depending on selection imposed by host genotypes. Wheat is highly diverse in resistance factors and likely recognizes a variety of different proteins produced by *Z. tritici* during infection. The deployment of different wheat resistance factors is also heterogeneous across the globe. Hence, if TE insertions contribute to adaptive modifications of effector genes, selection could act to influence TE frequencies in complex manners. In contrast, TE insertions near the two fungicide resistance loci were geographically limited. The high frequencies in Europe coincides with the early use of fungicides on the continent and widespread resistance gains (Hartmann et al. 2021). Similarly, some Oceanian populations have gained azole resistance recently (McDonald et al. 2019). Hence, it is conceivable that the geographically localized gains in TE frequencies are linked to azole resistance gains.

TEs insertions play an important role in shaping virulence and fungicide resistance evolution in fungal plant pathogens. Hence, identifying TEs dynamics near virulence and fungicide resistance loci provides fundamental knowledge on the evolutionary potential of the pathogen and can inform crop protection measures. Here, we identified a considerable number of TEs insertions from different TE families with geographically complex patterns. TE insertion activity is most likely ongoing with evidence for several singleton insertions at each of the analyzed loci. Such likely young TE insertions may also be maintained at low frequency in the populations due to purifying selection. In conclusion, our study provides a blueprint how to unravel TE dynamics at major loci contributing to adaptive evolution of a plant pathogen.

Methods

Isolates collection, culturing, and sequencing

Genome sequencing data was obtained from Feurtey et al. (2022). In brief, *Z. tritici* isolates were grown either on V8, yeast sucrose broth (YSB) or yeast malt agar (YMA) liquid or plates. DNA was extracted from lyophilized or frozen fungal tissue with QIAGEN kits (DNeasy Plant Mini Kit, QIAcube HT). TruSeq libraries were prepared from sheared DNA and libraries were successfully sequenced on an Illumina sequencing platform. For the collection provided by the Joint Genome Institute (see Feurtey et al. 2022), DNA was first extracted from single-spore isolates, and fragments were then treated with end-repair and A-tailing. Illumina-compatible adapters (IDT, Inc) were ligated using the KAPA-Illumina library creation kit (KAPA biosystems). PerkinElmer Sciclone NGS robotic liquid handling system was used for DNA library preparation for Illumina sequencing. Later, 200 ng of sample DNA was sheared to 600 bp using a Covaris LE220 focused-ultrasonicator. Sheared DNA fragments were selected based on their size by double-SPRI and then end repaired, A-tailed, and ligated with Illumina-compatible sequencing adaptors from IDT containing a specific molecular index barcode to distinguish each sample library. KAPA Biosystems' next-generation sequencing library qPCR kit was used to quantify the prepared libraries and then run on a Roche LightCycler 480 real-time PCR machine. The quantified libraries were then used for sequencing on the Illumina HiSeq sequencing platform utilizing a TruSeq paired-end cluster kit, v4. Sequencing of flow cells was performed on the Illumina HiSeq

2500 sequencer following a 2x150 indexed run using the sequencing kit HiSeq TruSeq SBS, v4. Overall, 1368 Illumina resequencing datasets were produced (Feurtey, Lorrain, et al. 2022). Details on the geographic origin, collection date, sequencing datasets and references are available for all included samples (Feurtey, Lorrain, et al. 2022).

De novo genome assembly

The quality of raw Illumina reads was checked using FastQC version 0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). In order to remove Illumina adapters and low-quality reads, reads were trimmed with Trimmomatic v.0.39 using the following filter parameters for trimming leading and trailing bases with a quality lower than 15 for the resequencing of 2020 and 10 for all previous resequencing. Additionally, sequences shorter than 50 bp were removed (Bolger et al. 2014). The novo draft assemblies were created using the software SPADES v.3.14.1 (Bankevich et al. 2012) with the "careful" method. Only assemblies (filtered to remove any contigs shorter than 1 kb) with fewer than 1600 contigs and a total length between 30 and 40 Mb were kept (Feurtey, Lorrain, et al. 2022).

TEs insertion detection and classification

To identify scaffolds containing the targeted genes, we first generated a BLAST database from each isolate draft assembly using the *makeblastdb* command (Altschul et al. 1990). We then used the faidx option of samtools v1.9 (Li et al. 2009) to extract matching scaffolds. Gene sequences of *AvrStb6*, *CYP51*, *MFS1* and *Avr3D1* were extracted from the reference genome IPO323 using the *faidx* function of samtools v1.9 (Li et al. 2009). BLASTn 2.12.0 (Altschul et al. 1990) was then used to identify the location of genes of interest on genomic scaffolds. The scaffold sequences matching each gene of interest were extracted with the function *faidx* of samtools v1.9 (Li et al. 2009). Prior multiple sequence alignment, we extended each sequence matching the gene sequence by 2000 bp in both directions in order to obtain intergenic sequences beyond the matching coding sequence. Then, we performed multiple sequence alignments for each locus across all different isolates using MAFFT version 7.407 with parameter --maxiterate 1000 (Katoh and Standley 2013). To characterize the detected TEs

insertions, RepeatMasker (Smit 2004) (http://www.repeatmasker.org/) was used to identify matching repeat regions and corresponding families based on the curated consensus sequences of TE families (Oggenfuss et al. 2021). Jalview version 2.10.5 (Waterhouse et al. 2009) was used to inspect multiple sequence alignments to ensure correct sequence alignments. We also used visual inspection if all possible TEs insertions across different isolates to identify singleton TEs. Singleton sequences of interest were screened for TEs using RepeatMasker as described above.

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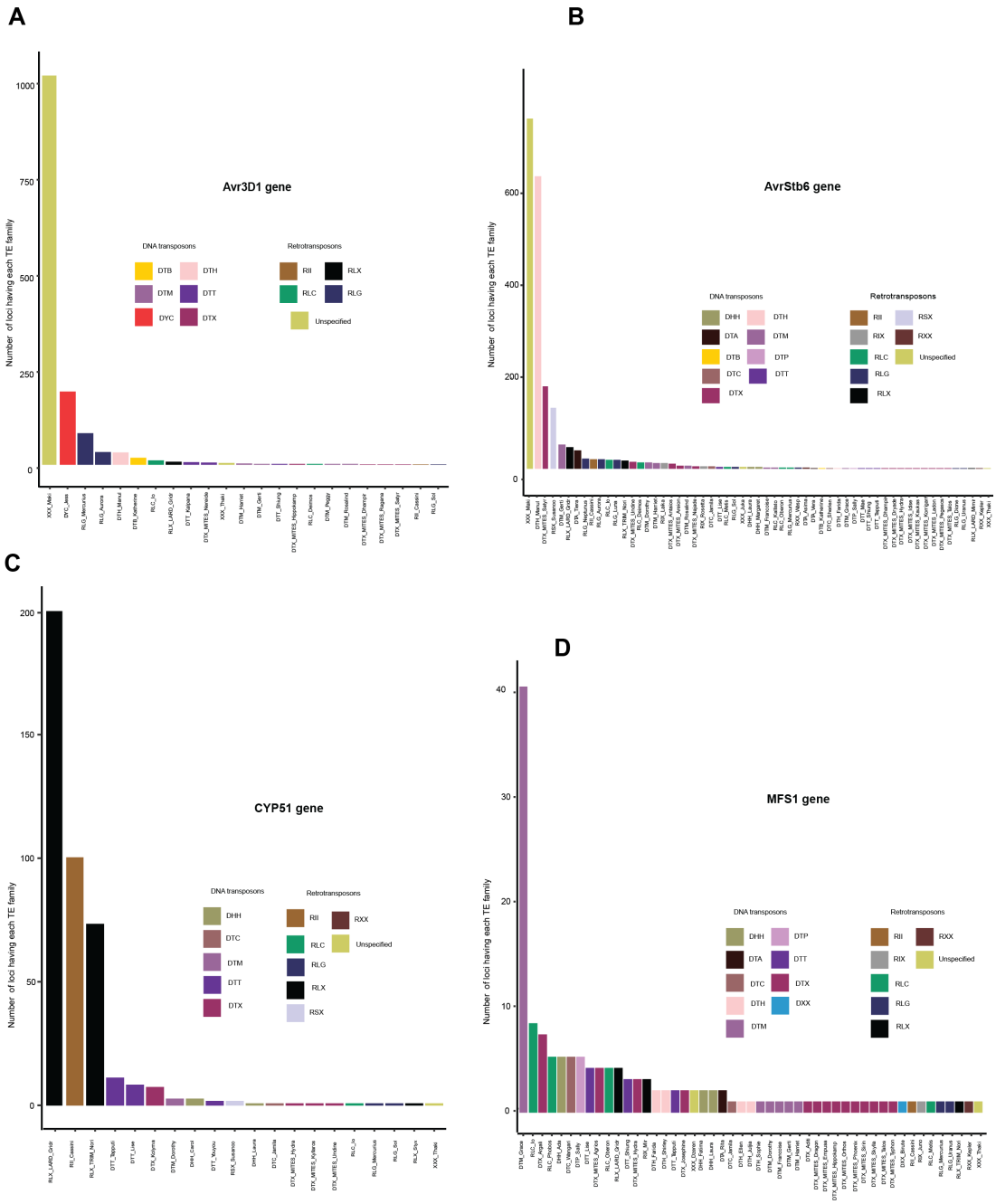
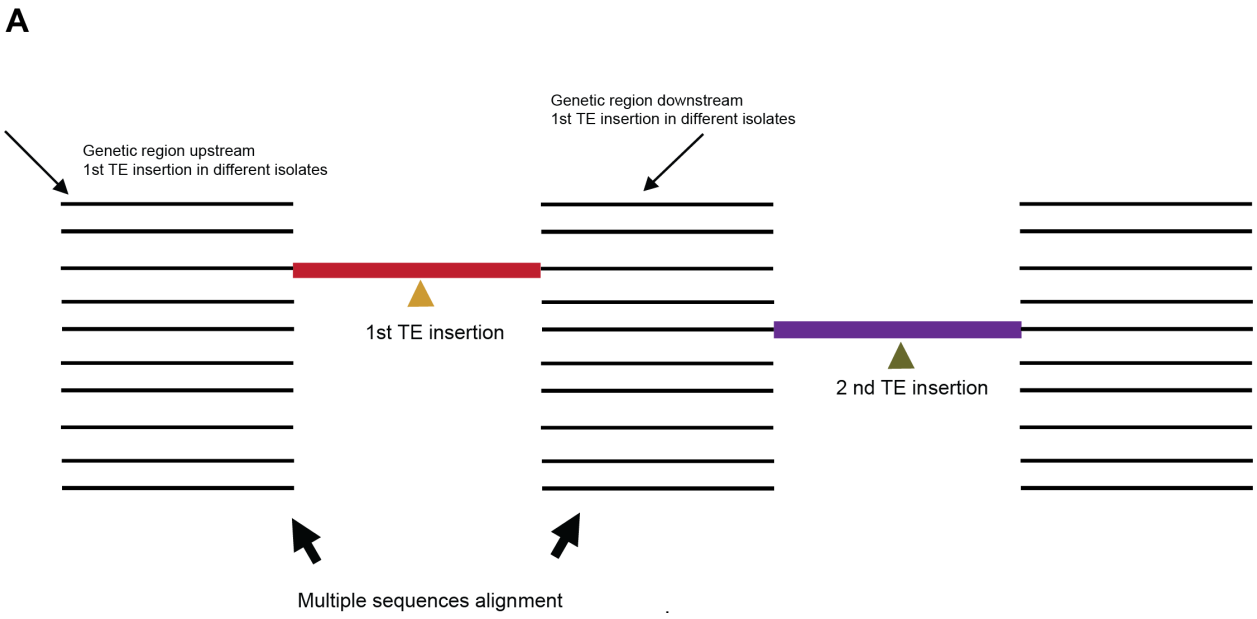
Supplementary Information

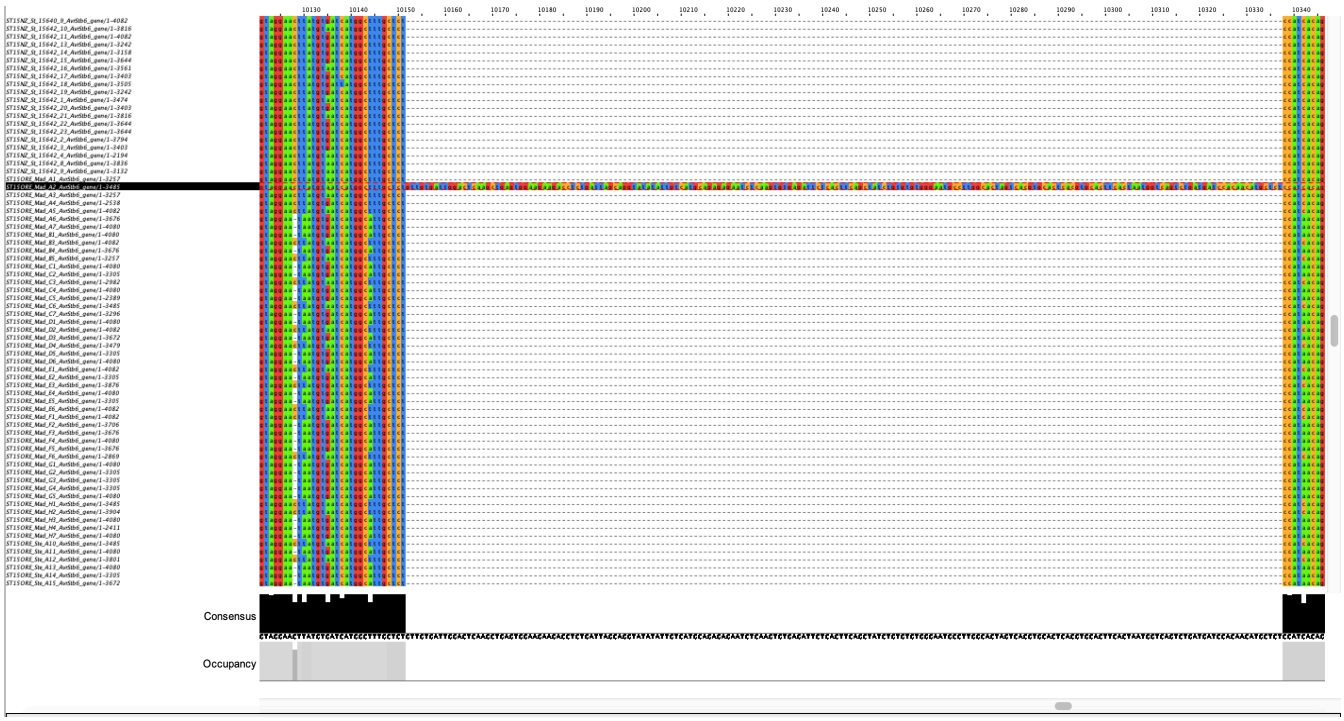
Supplementary Figures



Supplementary Figure S1: Number of loci carrying each TE family at different virulence and fungicide resistance gene loci. A) The count of loci carrying each TE family in *Avr3D1* locus. B) The count of loci carrying each TE family in *AvStb6* locus. C) The count of loci carrying each TE family in *CYP51* locus. D) The count of loci carrying each TE family in *MFS1* locus.



Supplementary Figure S2: Schematic overview of multiple sequence alignment to detect accurate TEs insertions.



Supplementary Figure S3: Multiple sequence alignment performed in Jalview to detect accurate TEs insertions.

Supplementary Table legends

(See Excel file)

Supplementary Table S1: Transposable element (TE) distribution in effector and fungicide resistance genes across different isolates.

Supplementary Table S2: Detected accurate TE insertions including singleton in effector and fungicide resistance genes across different isolates.

Supplementary Table S3: Singleton TE insertions in effector and fungicide resistance and their annotation in the reference genome IPO323.

General discussion and perspective

In this doctoral thesis I developed a monitoring tool to track the rapidly evolving wheat fungal pathogen called *Zymoseptoria tritici* (syn *Mycosphaerella graminicola),* the most damaging fungal pathogen of wheat in Europe, as well as investigating the outcomes of interactions that might occur between individuals within and outside the plant host. Additionally, I investigated transposable elements dynamics within the species that can be a key driver of adaptive evolution. In the **first chapter**, as global emergence of both fungicide resistant fungal strains as well as virulent strains overcoming the plant immune response is increasing rapidly. Crops of all types are, however, under constant threat from various fungicide resistant or and virulent emerging plant pathogens. Monitoring tools are important to achieve adequate control and detection of such emerging strains. We developed a new reliable microfluidics-based amplicon sequencing assay to timely and accurately multiplex hundreds of loci targeting virulence and fungicide resistance genes as well as randomly selected genome-wide markers to assess the genetic diversity of the fungus. Our system of monitoring combined with baiting trials is an especially powerful tool for two reasons: (i) this assay has showed a consistent amplification of different loci associated to virulence, fungicide resistance as well as genetic diversity across samples and can be used to monitor the emergence of relevant single points mutation, thus saving on time and resources ; and (ii) this assay has also been shown to perform well in different sample types originating from low input pure genomic DNA as well as mixed samples containing DNA both from multiple strain genotypes and the host (i.e. wheat plants) thus allowing us to identify cases of resistance, virulence emergence quickly with unprecedented sensitivity and accuracy. To our knowledge, this is the first study which uses a monitoring tool to simultaneously quantify fungicide resistance, virulence as well as assessing genetic diversity in the field. As evolution mediates genetic variation across the genome of strains within the same species and act in the generation of new genotypes to allow fungi to survive and reproduce. Hence, in nature, different strain genotypes of the same species can interact with each other and with their host. Interactions between strains of the same species might play a central role in shaping the disease severity and virulence evolution. The **second chapter**, was dedicated to intraspecific co-infections which are frequent in nature and plant host have been shown to be infected

by diverse conspecific species. Hence, we were able to provide a first study of comparison of the growth kinetics in monoculture vs. co-culture and investigating the outcome of intraspecific interactions outside and within a plant and how such intraspecific interactions can affect and change the fitness and virulence of strains outside and within a plant host respectively using a large sample size allowing providing a general overview of the different outcomes of such interactions. Besides, we could demonstrate that such outcomes are complex and were contingent on the environment and the genotypes of the competing and either in planta or *in-vitro* mixed interactions might lead to a change in growth and virulence of involved strains as well as modulating disease severity. This chapter highlights the importance of studying intraspecific pairwise interactions due their importance on impacting the disease severity and modulating of virulence and growth evolution of plant pathogens. The developed microfluidics assay in the **first chapter** is a powerful tool to study intraspecific interactions as it can be used to identify within species genetic variability of plant pathogens and therefore inform about possible prevalence of co-infection events. Due to the importance of transposable elements (TEs) in mediating adaptive evolution of fungi. The **third chapter** was dedicated to investigate the dynamics of transposable elements in important virulence and fungicide resistance related genes in 1012 isolates from a diverse global collection. We found that isolates carry a considerable amount of TEs insertions from different families and superfamilies with geographically complex patterns. As, many associations have been made between TEs and *Z. tritici* virulence and fungicide resistance, this chapter provides fundamental knowledge on the evolutionary potential of the pathogen resulting on the emergence of newly evolved pathogens , thus such information can inform crop protection strategies.

Monitoring tools to detect emerging fungal plant pathogens

Plant disease control repeatedly fails due to the constant evolution of pathogens. Fungi have evolved different strategies to evade plant host defenses and fungicide-based crop protection strategies (Mohd-Assaad et al. 2016; Nelson et al. 2018). Studies have shown that single nucleotide polymorphisms (*SNPs*) in virulence genes and genes encoding the targets of the fungicide compounds allow the pathogen to adapt to new environmental conditions by giving rise to new virulence and fungicide resistance pathogen genotypes (Mohd-Assaad et al. 2016; Corkley et al. 2022; Myint et al. 2022;

Shakouka et al. 2022). TEs insertions have also been shown to be related to virulence and fungicide resistance evolution (Bakkeren and Valent 2014; Omrane et al. 2017). Various monitoring tools have been used to identify newly evolving fungicide resistant pathogens ranging from in vitro sensitive fungicide resistance assays (Saville et al. 2015; Samils et al. 2021) to TaqMan assays based on fluorescently-tagged, allele-specific probes (Schleinitz et al. 2011). In general, such monitoring tools are labor-intensive, time consuming and limited to multiplexing low numbers of individual loci. Recently, a developed PacBio has been able to multiplex large number of loci related to fungicide resistance in *Z. tritici* (Samils et al. 2021). Moreover, virulence has been monitored through loop-mediated isothermal amplification (LAMP), an example includes the tomato wilt pathogen *Fusarium oxysporum f. sp. lycopersici (Fol)*. However, LAMP assays are expensive as result of costs of individual probes (Ayukawa et al. 2017). Whole genome sequencing (WGS) based techniques have also contributed to the mapping and characterization of virulence and resistance targets primarily through genome-wide association mapping (Mohd-Assaad et al. 2016; Zhong et al. 2017; Pereira et al. 2020; Hartmann 2022). A less time consuming, low-cost and accurate monitoring tool that combines detection of single point virulence mutations, fungicide resistance and genetic diversity has been developed (**Chapter 1**). This genotyping assay provided a good genomic coverage across most of all target loci. Additionally, it could robustly amplify across a range of sample types including low-input pure fungal DNA as well as mixed leaves samples (**Chapter 1**). TEs insertions might play an important role in fungicide resistance evolution (**Chapter 3**). Hence, this microfluidics-based amplicon sequencing assay has also been used to detect TEs insertion-based mutations related to fungicide resistance (**Chapter 1**). Even if the genotyping of TE insertions in the MFS1 promoter region did not work as expected as a result of high levels of sequence polymorphism, but this can be improved in the future to better capture inserted sequences. Furthermore, such developed targeted amplicon sequencing assays provide a highly sensitive technique to measure the frequency of specific alleles in plant infected leaves, this might detect diversity within *Z. tritici* strains that often result in co-infection events (**Chapters 1-2**). Hence, this tool can be used to identify mutations mediating evolutionary responses to changing environmental conditions as well as prevalence of possible co-infection events.

Plant pathogens can interact with each other leading to complex co-infection outcomes

In plant pathology, the majority of studies have been focused on single plant host infection (Figueroa et al. 2018; Pegg et al. 2019). However, in nature, co-infections are frequent (Susi et al. 2015). Co-infections can have various impacts on the host depending on the outcome of microbial interactions and host immune responses (Abdullah et al. 2017). Additionally, they might shape the evolution of pathogen virulence. Intraspecific co-infections are playing a key role in disease progression with fungal-plant pathosystem receiving most attention (Schürch and Roy 2004; Susi et al. 2015; Barrett et al. 2021). However, deeply studying pairwise interactions within and outside the host would be very important as they are key modulators of disease outcomes. Field populations of *Z. tritici* show high phenotypic and phenotypic diversity worldwide due to several factors including frequent sexual reproduction, large populations, and significant gene flow among populations (Zhan et al. 2005). Moreover, within pathogen species have diverse virulence degrees (Schürch and Roy 2004; Barrett et al. 2021). Therefore, co-infection of wheat leaves by multiple strains of *Z. tritici* are frequent in the field (Linde et al. 2002). The virulence of *Z.tritici* appears unaffected by co-infections though (Schürch and Roy 2004; Barrett et al. 2021). Besides, the competitive advantage of individual strain genotypes for transmission is not associated with virulence among the strains (Bernasconi et al. 2022). Additionally, pairwise interactions can produce divergent outcomes either in culture conditions or within a plant host depending on conspecific pairing genotypes (**Chapter 2).** Hence, such finding raises question about the mechanisms involved in such competitive interactions. Moreover, highly virulent strains are not necessarily the most competitive outside their host (**Chapter 2)**. It has also been found that the outcomes of mixed interactions outside the host are not a good predictor of those performed in planta (**Chapter 2)**. The divergence in the outcomes of competitive interactions make predicting co-infection damage more challenging. However, systematic screens of pairwise interactions combined with genetic analyses will help build more informed models of pathogen evolution on crops and other plants.

TEs insertions mediate plant pathogen adaptive evolution

TEs have been shown to play an important role in shaping pathogens evolution. However, screening of TEs dynamics in virulence and fungicide resistance genes would be very important. TEs content is variable among different *Z. tritici* strains (Badet et al. 2020). Furthermore, accessory chromosomes contain the higher proportions of TEs than the core chromosomes. Effector genes and fungicide resistance associated genes are known to be located in TE rich region. A TE insertion into the promotor region in the MFS1 gene coding MFS1 transporter causes overexpression and multidrug resistance in *Z.tritici* (Grandaubert et al. 2014; Soyer et al. 2015; Omrane et al. 2017; Torres et al. 2020). This study is the first screening of TEs in effector genes at the global level. The variation in TEs insertions between fungicide resistance and effector associated genes might be due to various forces that shape TE insertion site preferences, weaker purifying selection or positive selection on beneficial insertions. Analysis of the most frequent TEs showed that dynamics of TEs at the virulence and fungicide loci found in a single field population also expanded to global population (**Chapter 3**). Furthermore, inserted TEs belong to various TEs families indicating that multiple TE families were active at least in recent history **(Chapter 3)**. TE families and superfamilies may have insertion site preferences. Some TE superfamilies preferentially insert into coding regions or near transcription start sites (Gilly et al. 2014). Rare TEs (identified as called singletons) show most likely ongoing insertion dynamics of TEs creating new insertions **(Chapter 3)**. Whether some TE insertions near virulence or fungicide resistance genes affect phenotypic traits could be assessed using molecular genetics tools (see e.g. Omrane et al. 2017) or genome wide association mapping studies (GWAS) based on trait variation.

Plant pathogens evolve to overcome crop protection-based strategies. A constant monitoring of emerging pathogens would be important for a sustainable crop management. Here, we developed a targeted amplicon sequencing assay that shows a consistant amplification of different loci to track virulence and fungicide resistance evolution across different isolates. This might contribute to providing information about the adaptive potential of plant pathogens in the fields and their evolutionary history as well. Detection of emerging plant pathogens can be challenging for pathogens with low DNA input or originating from mixed samples. Here, the newly developed monitoring tool will overcome this issue

as it can perform well, either in mixed samples or in low input DNA samples. Nowadays, the worldwide frequent application of fungicide makes a rapid rise in resistant plant pathogen genotypes. Therefore, this developed assay can simultaneously genotype a wide range of loci making tracking fungicide resistant fungi less laborious and faster. Whole genome sequencing (WGS) allows the availability of several mutations associated with the emergence of several newly evolved plant pathogens. Hence, this genotyping assay can be used to detect available mutations associated with virulence, fungicide resistance as well as assessing genetic diversity of wide range of plant pathogens to improve crop management strategies.

Despite the fact that intraspecific co-infections are a very common in nature and might modify the virulence of the pathogen. Studying the outcomes of such interactions within and outside of the plant host as well as change of virulence and growth that might be associated is still poorly studied. Previous studies have shown that co-infections of plants by different plant pathogen can significantly alter infection severity and can cause varying levels of damage to the host depending on the outcome of microbial interactions and host immune responses. Most of these studies were performed with small number of competing genotypes and therefore providing little information about their all-possible consequences. This thesis investigates the outcomes of pairwise interactions outside and within a plant host using a wide range of genotypes. This can allow us to provide a big picture of mixed interactions consequences either on plant host or on the evolution of virulence and growth. Besides, due to the complexity of the outcomes of such interactions. It is clear that creating a universal theoretical model to predict good competitors outside and within a host, as well as virulence and fitness change would be crucial. Hence, our finding would contribute to create a statistical model to predict mixed interactions outcomes.

Studies on TEs play an important role on understanding the adaption of plant pathogens to various environmental conditions. However, most of these studies focused on individual insertions whereas, studying global populations TEs dynamics would be important to assess the evolutionary history of the plant pathogens and therefore allowing a better crop disease management. Hence, in this thesis, we

provided information about TEs insertions located near to important virulence and fungicide resistance associated genes loci but at the same time open new questions in the role of these detected TEs in the virulence and fungicide resistance evolution. This might be achieved in the future by using molecular tools or genome wide association studies (GWAS). Besides, our finding also opens questions about understanding the forces shaping such within species evolution.

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