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DOCTORAL THESIS

Genetic analysis of resistance in *Triticum* spp. to newly emerging races of wheat stem rust

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Declaration

I hereby declare that my doctoral thesis **Genetic analysis of resistance in *Triticum* spp. to newly emerging races of wheat stem rust** has not been submitted for any other degree to this or any other university. I declare that this thesis is solely my own work unless otherwise referenced, declared, or acknowledged.

In Prague, 20.3.2024

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Ing. Ondřej Zelba

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Abstract (English)

Stem rust of wheat (*Triticum* spp.) is one of the most significant fungal diseases in agriculture. Its research and breeding for resistance pays special attention to the tropics and subtropics, where yield losses are most severe. In recent years, however, the disease has also been reappearing in the European continent through incursions of novel races of the pathogen (*Puccinia graminis* Pers.) from tropics and subtropics and the pathogen population is adapting to the mild climate. This thesis analyses the basis of genetic resistance of cultivars grown in the Czech Republic in light of virulence of novel pathogen races. A panel of locally grown wheat cultivars is tested for presence of resistance genes using molecular markers. Results of marker assays are compared to field trials and greenhouse phenotypic tests using characterized pathogen races. Additionally, several Kompetitive allele-specific PCR (KASP) markers linked to resistance genes *Sr8a* and *Sr11* are tested on local wheat germplasm for the first time to validate them for detection of the resistance genes. Furthermore, adult plant resistance phenotyping is undertaken with a small subset of cultivars to describe their resistance into more detail. Quantification of disease severity is done using visual assessment, image analysis with machine learning, and assessment of fungal biomass based on chitin quantification. Results of marker assays suggest that gene *Sr38* is present most frequently (63.8%). While cultivars with this gene show lower disease severity in the non-race specific field trials in previous years, isolates collected in 2020 season are mostly virulent to it. Resistance genes *Sr31* and *Sr24* are present with 10.3% and 13.8% frequencies and are still effective towards present races. Cultivars with a combination of two out of all the tested genes show best results in field trials compared to cultivars with one or no detected resistance genes. Adult plant phenotyping demonstrated that there is adult plant resistance in some cultivars which is not otherwise discernible. Image analysis using Trainable Weka Segmentation was performed successfully with both seedling and adult plant samples and usage of this easy method of quantifying symptoms of rust disease is validated here. KASP markers linked to genes *Sr8a* and *Sr11* show conflicting results and further phenotypic tests will have to be done to prove their applicability. This thesis uses molecular methods and disease phenotyping to provide an up-to-date overview of stem rust resistance of wheat in Czech cultivars, useful for breeders, farmers, and future researchers.

Keywords: stem rust, wheat, resistance genes, fungal pathogens, molecular markers

Abstract (Czech)

Černá rzivost trav je jednou z nejvýznamnějších houbových chorob v zemědělství. Její výzkum a šlechtění pšenice na odolnost k této chorobě je převážně zaměřen na tropické a subtropické oblasti, kde jsou způsobené ztráty na výnosech nejvýznamnější. V současné době se však černá rzivost trav vrací na evropský kontinent. Bylo zaznamenáno šíření tropických ras patogenu (*Puccinia graminis* Pers.), které se se úspěšně adaptují na mírnější klimatické podmínky. Tato práce se zaměřuje na studium rezistence u odrůd pšenice pěstované v České republice zejména v souvislosti s nově detekovanými patotypy. Geny rezistence byly zjišťovány v panelu odrůd pomocí molekulárních markerů. Výsledky byly srovnány s infekčními testy rezistence v polních podmínkách a dále fenotypovými testy rezistence klíčních rostlin s použitím popsaných ras patogenu. Dále bylo na místních odrůdách testováno několik Kompetitive allele-specific PCR (KASP) markerů pro použití k detekci genů *Sr8a* a *Sr11*. Další fenotypové testy byly provedeny také na dospělých rostlinách, na malém vzorku odrůd, pro bližší popis jejich rezistence. Kvantifikace stupně napadení byla provedena vizuálním zhodnocením, obrazovou analýzou se strojovým učením, a měřením obsahu chitinu ve vzorcích. Výsledky analýz naznačují, že gen rezistence *Sr38* je přítomen v českých odrůdách nejčastěji (63.8 %) a přestože v polních pokusech byl stupeň napadení u odrůd nesoucí tento gen nižší, rasy přítomné v roce 2020 jsou k němu již převážně virulentní. Geny *Sr31* a *Sr24* jsou přítomny v 10.3 %, resp. 13.8 % odrůdách a jsou nadále efektivní proti současným rasám. Odrůdy s nalezenou kombinací dvou z testovaných genů rezistence byly v polních pokusech podle očekávání nejlépe hodnoceny. Fenotypové testy na dospělých rostlinách ukázaly, že odolnost dospělých rostlin je v některých odrůdách přítomna a jinými testy nelze zjistit. Obrazová analýza se strojovým učením byla úspěšně provedena s klíčními i dospělými rostlinami a je zde prokázána jako jednoduchá metoda kvantifikace symptomů rzivostí pšenice. KASP markery vázané na geny *Sr8a* a *Sr11* vykazují neprůkazné výsledky a pro jejich další použití musí být provedeny navazující fenotypové testy. Tato disertační práce přináší aktuální poznatky v oblasti rezistence pšenice v českých odrůdách, a to ve světle nově se vyskytujících ras patogenu černé rzivosti trav. Výsledky mohou být uplatněny ve šlechtění, praxi i budoucím výzkumu.

Klíčová slova: černá rzivost trav, pšenice, geny rezistence, houbový patogen, molekulární markery

Table of contents

List of figures

List of tables

List of abbreviations

AFLP	Amplified fragment length polymorphism
Avr	Avirulence
BC	Backcrossing
bp	Base pairs
BYDV	Barley yellow dwarf virus
CAPS	Cleaved amplified polymorphic sequence
CC	Coiled-coil
CDC	Cell division control
cDNA	Complementary DNA
CIMMYT	Centro Internacional de Mejoramiento de Maíz y Trigo (International Maize and Wheat Improvement Center)
CRI	Crop Research Institute, Prague
CRISPR	Clustered regularly interspaced short palindromic repeats
DH	Double haploid
EMS	Ethyl methanesulfonate
ETI	Effector-triggered immunity
f. sp.	forma specialis
GMO	Genetically modified organism
HR	Hypersensitive reaction
ICARDA	International Center for Agricultural Research in the Dry Areas
ISSR	Inter-simple sequence repeat
KASP	Kompetitive allele-specific PCR
LRR	Leucine rich repeat
MAS	Marker-assisted selection

MQTL	Meta-QTL
NLR	Nucleotide-binding domain leucine-rich repeat
PAMP	Pathogen-associated molecular pattern
PBC	Pseudo black chaff
PCR	Polymerase chain reaction
Pgt	*Puccinia graminis* f. sp. *tritici*
Pst	*Puccinia striiformis* f. sp. *tritici*
PTI	PAMP-triggered immunity
Pt	*Puccinia triticina*
qPCR	Quantitative PCR
QTL	Quantitative trait locus
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
SCAR	Sequence-characterized amplified region
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeat
STS	Sequence-tagged site
TGW	Thousand grain weight
ÚKZÚZ	Ústřední kontrolní a zkušební ústav zemědělský (Central Institute for Supervising and Testing in Agriculture)
USDA	United States Department of Agriculture
WAC	WGA chitin assay
WGA-FITC	Wheat germ agglutinin, FITC-conjugate

1. Introduction

Wheat production around the world is hindered by yield loss due to various abiotic and biotic stresses. Among the biotic stresses, fungal diseases are some of the most damaging (Singh et al. 2016; Savary et al. 2019). Stem rust of wheat (caused by *Puccinia graminis* f. sp. *tritici*) is a disease that is rapidly spread by spores and its causal pathogen develops newly virulent races through several means (Huerta-Espino et al. 2014). Due to its sudden and very destructive outbreaks, stem rust has been a major focus of scientists and breeders. Overall, it is one of the most studied diseases in agriculture.

In the second half of the twentieth century, this disease was mostly considered to be under control. It was partly due to the eradication of the secondary host, barberry (*Berberis vulgaris*), in the temperate areas (Peterson 2018; Barnes et al. 2020). This prevented the completion of the pathogen's life cycle and quick spread of new virulent races into the fields. The second successful control measure was breeding for resistance. The international breeding efforts provided cultivars with resistance that was at that time considered durable and prevented large-scale epidemics, even in the vulnerable tropical areas (Rajaram et al. 1988; Simmonds 1988).

Stem rust reclaimed the spotlight right at the end of the twentieth century when a new, highly virulent race appeared in Uganda, later to be called Ug99 (Pretorius et al. 2000). This race caused problems in several African countries because it broke down a resistance conferred by gene *Sr31*, which was widely deployed in wheat cultivars in the area. With nothing to stop the fungus, it propagated quickly, spread through Africa and further to middle east. New races that were derived from the original Ug99 broke down resistance of further genes along the way (Singh et al. 2011).

In recent years, incidence of stem rust has also been increasing on the European continent, even in places where it had not been present for decades. Outbreaks in Sicily (Bhattacharya 2017) and Germany (Olivera Firpo et al. 2017) were especially alarming, but novel races appeared as far north as Ireland and Sweden. Genotypic studies showed that collected pathogen samples belong to genetic groups that originated outside of the continent, in tropical and subtropical areas (Patpour et al. 2022a). They are incursions with novel virulence patterns that seem to be adapting to the mild climate of Europe. Genetic resistance to this disease is now deficient in European agricultural production, partly because it has been considered well in hand for a long time.

This import of new races together with increased incidence warrant the need to refocus attention on stem rust resistance in cultivars grown in Europe, including Czech Republic. This study aims to first provide a concise but detailed literature review on stem rust concentrating on tropical and European regions and exchange of inoculum between them. In its practical part, the thesis provides

data on stem rust resistance in cultivars grown in the Czech Republic in current times. It aims to elaborate the efficiency of deployed resistance genes considering novel races from the tropics. The integration of molecular markers together with disease phenotyping provides information that is relevant not only for breeders and farmers on the local level, but also fills a gap in knowledge of worldwide defence strategies against a common enemy.

2. Literature review

2.1. Wheat rusts

Wheat rusts are diseases of wheat caused by fungal pathogens of the order Pucciniales. They are obligate biotrophic fungi with a complicated life cycle that may include more than one plant host. During the asexual phase their urediospores propagate indefinitely on the cereal host and their spores spread across fields to infect more and more plants, causing significant yield losses.

Three important species cause rust on wheat. It is *Puccinia triticina* Eriks. causing leaf rust, *P. graminis* f. sp. *tritici* causing stem rust, and *P. striiformis* f. sp. *tritici* causing stripe rust. They are often abbreviated *Pt*, *Pgt*, and *Pst*, respectively. The best defence against the diseases is growing resistant cultivars.

2.2. Biology of wheat rust pathogens

Wheat rust fungi belong to the order Pucciniales (Position in classification: Pucciniaceae, Pucciniales, Incertae sedis, Pucciniomycetes, Basidiomycota, Fungi). The order Pucciniales also comprises of roughly 8,000 other species of obligate parasites of vascular plants (Kirk et al. 2008). It is the largest group of plant pathogens, and its species are usually highly specialized in their host range.

Wheat rust fungi have a heteroecious lifecycle during which they produce five different types of spores (Hiratsuka & Sato 1982). They infect different host plants for sexual and asexual propagation. For all three of the wheat rusts, the asexual propagation takes place in a grass host (Poaceae). The range of grass hosts reaches far outside the group of cereal crops, in fact, wheat rusts have been pathogens on grasses before cereals crops came to existence and then adapted to them with the onset of agriculture. In case of *Pst*, the range of grass hosts includes more than 100 species (Chen & Kang 2017). The range of hosts for sexual propagation is much more limited. In case of *Pst* and *Pgt*, this part of life cycle mostly occurs on common barberry (*Berberis vulgaris* L.) (Roelfs 1985; Jin et al. 2010), while for *Pt* it occurs on *Thalictrum* spp. These are shrubs that are completely unrelated to the asexual hosts in the Poaceae family, implying that the pathogens must have co-evolved with both the hosts (Leppik 1961; Wahl et al. 1984; Burdon & Laine 2019).

The five types of spores that are produced are basidiospores, urediospores, teliospores, pycniospores, and aeciospores (Roelfs 1985; Kirk et al. 2008). Here, the spores of *P. graminis* f. sp. *tritici* are shortly described before moving on to the life cycle of rusts in the following paragraphs. Sexual propagation occurs on a secondary host, where aeciospores are produced, which can

inoculate the primary grass host. They are dikaryotic, cylindrical, approximately 16-23 x 15-19 µm large (Roelfs 1985). Urediospores are produced by asexual propagation on the primary grass host and can infect the grass host again. They are dikaryotic, elongated, with a spine-covered surface, ranging between 26-40 x 16-32 µm in size (Roelfs 1985). Anikster et al. (2005) report 28.3 x 17.5 µm as the mean urediospore size. The spines covering surface of urediospores are approximately 0.4 µm x 0.8 µm (Thomas & Isaac 1967). Teliospores are produced in telia on the grass host and undergo karyogamy to produce basidiospores. They are two-celled spores with pedicels and caps formed by apical wall thickening, 49.5 x 19.8 µm (Anikster et al. 2005). Basidiospores are small (9.83 x 6.46 µm), asymmetrical, and short-lived. They infect the secondary host, where pycnia are formed on the top side of a leaf and pycniospores are produced. Pycniospores are the smallest spores, pear-shaped, with thin walls, approximately 3.65 x 2.16 µm in size (Anikster et al. 2005). Pycniospores and receptive hyphae of opposite mating type fuse and aecium is formed on the bottom side of the leaf. There, aeciospores are produced that are released and can inoculate the primary grass host again. Aeciospores are cylindrical, ranging between 16-40 x 14-32 µm in size (Roelfs 1985). The spores that inoculate the cereal hosts (aeciospores and urediospores) are dikaryotic and as with other basidiomycetes, the dikaryotic phase is dominant. It is also the most important part of the life cycle for humans since it is the cause of economic losses in agriculture.

The infection process on grass host begins with contact of aeciospore or urediospore with epidermis of plant. A film of water on the surface of plant tissue is necessary for the spore to germinate and form a germ tube that elongates across the venation until it reaches a stoma. In case of *Pgt* and *Pt*, an appressorium is formed at the entrance of stoma, in case of *Pst*, appressorium is usually not formed. Stoma is then penetrated by a penetration peg and a structure called substomatal vesicle is formed below stoma in the mesophyll. Infection hyphae develop and prolong in the apoplast until a haustorial mother cell is formed at the distal end. The infection hyphae are compartmentalized by septa with centrally located pores (Ehrlich et al. 1968). The haustorial mother cells are clearly separated from the infection hyphae by septa and contain smaller nuclei and thicker, more layered cell walls (Chong et al. 1985).

Haustoria are then formed which establish contact with the plant mesophyll cells and enable the uptake of nutrients. Haustoria are connected to the haustorial mother cell by a short haustorial neck. The structure of haustoria is adapted to make the contact area between the fungus and the host cell large by forming an invagination in the plant cell wall and establishing an extrahaustorial matrix between its wall and the extrahaustorial membrane derived from the plant cell wall (Chong et al. 1986). This is crucial for effective transport of nutrients (Garnica et al. 2014). In case of *Pst* it has been shown that haustoria form apical branches which make the contact area even larger (Sørensen

et al. 2017a). Haustoria are typical structures of obligate parasitic fungi, and they are critical for the exchange of proteins and nutrients that are means of communication between pathogen and host (Staples 2001; Voegele & Mendgen 2003; Bushnell 2003). These exchanges can determine compatibility or incompatibility of the interaction because of many secreted effector proteins that can be recognized by receptor proteins of the host (Garnica et al. 2014).

The infection cycle completes in about 10 – 11 days when uredia are formed close to the leaf surface and production of many urediospores causes eruption of the epidermis and the spores are then released and can begin another round of infection on the grass host. The erupted pustules on the leaf surface are visible with naked eye and are the typical symptom of the disease (Figure 1). In case of *Pgt*, those are darker brown than in case of *Pt,* which is a reason why the disease was also called black rust while disease caused by *Pt* was alternatively called brown rust. Uredia of *Pst* are aligned in typical yellow stripes which gave the disease its name stripe rust or alternatively yellow rust.

Figure 1: Typical manifestation of severe stem rust disease on wheat. Pustules abounding with urediospores rupture the epidermis of a susceptible plant from the inside and spores are released into environment (Photo by author, taken during field trials in Crop Research Institute, Ruzyně, Prague)

Eventually, the fungus in the grass host creates the resting spore stage, the telia and teliospores. In temperate regions this happens in autumn. Teliospores are dikaryotic, thick-walled spores that are not released but stay dormant on the grass host. In spring they form a basidium and basidiospores with two haploid nuclei are ejected from the host. These basidiospores are then able to germinate on the surface of the secondary host. This needs to happen at the time when the shrubs are budding and creating new leaves, which is an ideal phase for inoculation by basidiospores.

On leaves of barberry in case of *Pgt* and *Pst*, or *Thalictrum* in case of *Pt*, the infection manifests itself by formation of flask-shaped pycnia on the upper side of the leaf. In addition to common

barberry, *Pgt* can infect several other *Berberis* species and hybrids and some Mahonia species as well (Roelfs 1985). Pycniospores are produced surrounded by pycnial nectar and they act as a male gamete in the sexual reproduction cycle. Receptive hyphae in the pycnia act as the female gamete. A pycniospore needs to connect with a receptive hypha from a pycnium of an opposite mating type. The spread of pycniospores is presumably facilitated by insect or rain. A hypha containing two to three nuclei (sometimes more) then migrates until it reaches a protoaecium which is below the pycnia on the bottom side of the leaf. A cluster of hyphae with different amounts of nuclei is present and and as aecium matures, aecial cups are formed where dikaryotic aeciospores are produced in chains (Allen 1929, 1930) (Figure 2). By this time the leaf epidermis is ruptured and the aecial cups can break and release many aeciospores into air. Aeciospores are then able to inoculate the grass host (Leonard & Szabo 2005; Bolton et al. 2008).

Figure 2: Aecia of Puccinia graminis with broken aecial cups formed on abaxial side of barberry leaves (Photo by author, taken in Prague, Děvín in 2020)

2.3. Classification of *Puccinia graminis*

2.3.1. Subspecies and formae speciales

There is a significant physiological variation inside the *P. graminis* species. The general host range for the asexual (uredial) life stage includes thousands of gramineous species, but individual strains of the fungus are adapted to different range of those species. This led to efforts to classify the fungus on a sub-species level. Czech mycologists proposed distinction between *P. graminis* subsp. *graminis* and *P. graminis* subsp. *graminicola* (Urban & Marková 1983, 2009). According to this

classification, the subspecies *graminis* has adapted to infect cultivated cereals but cannot overwinter in temperate Central Europe. It was not found to complete its sexual cycle on barberry or mahonia (*Mahonia* spp.) in Czech Republic (studies were undertaken in former Czechoslovakia). The second subspecies, *graminicola*, is not able to infect cultivated wheat (Urban 1967), has a much wider host range among wild grasses, has significantly smaller urediospores, and completes its life cycle on barberry in the same region. It is understood that ssp. *graminicola* is the ancient form adapted to non-cultivated species, from which the ssp. *graminis* evolved by adapting to agricultural crops. Correct distinction between those subspecies needs to be confirmed by measuring spore sizes (Urban & Marková 2009). Furthermore, making a distinction between the subspecies based on their ability to complete sexual cycle on barberry cannot be extended outside of the region of former Czechoslovakia, where the studies were undertaken, because the conditions could differ elsewhere (Wahl et al. 1984).

The distinct adaptation to different hosts led to establishment of *formae speciales (ff. spp.)*, another division in taxonomy below the species level (Anikster 1984). Notably, this classification is based on physiology and the compatibility between pathogen and host rather than morphology. The precise distinction between the *ff. spp.* is usually not clear as the exact host range of the strains is very laborious to study. At least two forms are distributed globally since they are adapted to globally cultivated species: *P. graminis* f. sp. *tritici* on wheat and barley and *P. graminis* f. sp. *secalis* on rye. Some other *ff. spp.* described are *avenae*, *agrostidis*, and *poae* (Johnson 1949; Anikster 1984). This differentiation is based on the most common cereal host. The cultivated wheat-infecting *forma specialis* that causes most damage in agriculture, *P. graminis* f. sp. *tritici* falls within the *P. graminis* subsp. *graminis*. In this thesis, the abbreviation *Pgt* will be used from this point onwards.

2.3.2. Physiological races

While the distinction between *ff. spp.* is guided by adaptation to host species, there is further variation in reaction to various genotypes within the host species. This is especially significant in agriculture, because a single *Pgt* genotype may provoke different reactions on multiple host genotypes of wheat. When using a set of known lines of wheat, we can differentiate between individual physiological races of the pathogen. The distinction is made by inoculating differential set of host lines and recording the virulence / avirulence pattern, i.e., compatible / incompatible reactions. Physiological race (biotype or group of biotypes), while not officially recognized as a taxonomy unit, is still used to differentiate variations inside the *formae specialis* based on physiology and host-pathogen interaction (Roelfs 1984).

It has been known since the early 1900s, that different races of rusts exist within species. A first guide to distinguish races of *Pgt* was published by Stakman and Levine (1922). It instructed to

inoculate differential wheat lines with samples of *Pgt* and then determine the stem rust infection types using the Cobb scale (Peterson et al. 1948). Similar procedure is still used today, 100 years later, but today near-isogenic lines (NILs) are used as differentials (Knott 1990). It is a set of lines with a described genetic resistance. A system of nomenclature was proposed for *Pgt* physiological races in the 1980s that used three-letter codes for race designations (Roelfs & Martens 1988), but as the number of differentials grew to 20, five-letter codes are now used (Jin et al. 2008). Recording the high or low infection types on each line provides the five-letter code (Table 1). Using this system, a hypothetical strain virulent to all the differentials would be termed TTTTT, a strain avirulent only to *Sr24* would be termed TTTTK and so on. This nomenclature has been widely adapted by many institutions worldwide and works as a common language between researchers, but more lines can be used to achieve even higher resolution. In some studies, as many as 47 lines were used to define the isolates more precisely (Guo et al. 2022). Some scientists suggest adding more letters to the system to be able to differentiate new races in the ever-evolving pathogen population (Fetch et al. 2021).

Table 1: Differential lines used for Pgt nomenclature and an aid to deduct the letter code. L stands for low infection type (0-2 on Cobbs scale) and H stands for high infection type (3-4 on Cobbs scale). Letters derived from virulence profiles on the five differential sets are then concatenated into a lettter code (such as TTKSK). Adapted from Roelfs & Martens (1988), and Jin et al. (2008).

Five sets of differential lines:				
Set 1	*Sr5*	*Sr21*	*Sr9e*	*Sr7b*
Set 2	*Sr11*	*Sr6*	*Sr8a*	*Sr9g*
Set 3	*Sr36*	*Sr9b*	*Sr30*	*Sr17*
Set 4	*Sr9a*	*Sr9d*	*Sr10*	*SrTmp*
Set 5	*Sr24*	*Sr31*	*Sr38*	*SrMcN*
***Pgt* letter code:**				
B	L	L	L	L
C	L	L	L	H
D	L	L	H	L
F	L	L	H	H
G	L	H	L	L
H	L	H	L	H
J	L	H	H	L
K	L	H	H	H
L	H	L	L	L
M	H	L	L	H
N	H	L	H	L
P	H	L	H	H
Q	H	H	L	L
R	H	H	L	H
S	H	H	H	L
T	H	H	H	H

2.3.3. Phylogenetic clades

Molecular genetics enables us to study relationships between pathogen isolates, infer phylogenies and group individuals from a population inside clades. Today, this is most commonly done on *Pgt* samples from around the world using either SSR markers or sequencing data. Knowledge of genetic relationships allows to study evolution of new virulence and enables monitoring of population

shifts. Up to now there are eight defined clades which can be further differentiated into sub-groups (Patpour et al. 2022). Although there is a clear relationship between phylogeny of isolates and their virulence profiles, it should be noted that a virulence profile based on scoring on 20 differentials could arise in different evolutionary lineages of the pathogen independently. To properly classify an isolate, both phenotyping and genotyping should be undertaken. Table 2 serves as an example of proper classification of pathogen isolates, with information on year and location of sampling, physiological race, clade, and ID of the isolate.

Table 2: Examples of P. graminis samples classification based on their physiological race and phylogeny.

Year	Location	Physiologic race	Phylogenetic clade	ID of isolate	Reference
2004	Kenya	TTKSK	I (Ug99 clade)	04KEN156/04	*Szabo et al. (2022)*
2014	Kenya	TTKTT	I (Ug99 clade)	14KEN58-1	*Newcomb et al. (2016), Guo et al. (2022)*
2014	Ethiopia	TRTTF	III-A	14ETH123-1	*Szabo et al. (2022)*
2017	Ethiopia	TTRTF (*Sicily race*)	III-B	17ETH306-2	*Szabo et al. (2022)*
2013	Germany	TKTTF (*Digalu race*)	IV-A.1	13GER15-2	*Olivera Firpo et al. (2017)*
2013	Denmark	TKTTF (*Digalu race*)	IV-A.2	DK185a/13	*Patpour et al. (2022)*
2017	Hungary	TKTTF (*Digalu race*)	IV-B	HR210b1/17	*Patpour et al. (2022)*
2019	Czech Republic	RFCNC	VIII	CZ33_19	*Patpour et al. (2022)*

2.4. Evolution of virulence

Cereal rust pathogens are dikaryotic fungi with a very plastic genome. *Pgt*'s ability to rapidly develop new races with novel virulence is enabled by several means. During asexual propagation, (i) mutations can occur and modify the genes by point mutations or indels, (ii) exchange of genetic information from one nucleus to another in one race is possible. New races can also arise from (iii) somatic fusion, the exchange of two intact nuclei from two different races (Figueroa et al. 2020). The frequency and importance of somatic fusion is not yet well understood in rust fungi, but genome sequencing has shown that some important races such as Ug99 have resulted from it (Li et al. 2019a). Alternatively, new genetic variations can also emerge from sexual propagation, which requires the alternative host, *Berberis vulgaris*. Since this method of propagation is highly dependent on climatic conditions and on presence of the alternate host, it is geographically limited.

In some areas the sexual propagation does not play a role while other areas are a continuous source of new races (Groth & Roelfs 1982; Jin et al. 2014).

In conclusion, a location where the pathogen propagates only asexually is bound to have a population with low genetic variability, where new virulence can occasionally arise via stepwise mutations. These races will probably break down one resistance gene of wheat at a time and the rate of evolution will depend on the amount of disease which is allowed on crops. On the other hand, a location with abundant sexual propagation on barberry will have a *Pgt* population with much higher genetic variability and a much higher variability of virulence patterns due to crossing-over between races.

2.5. Stem rust in agriculture

In general, it can be said that wheat rusts occur in all areas where wheat is grown. The yield and financial losses caused by cereal rusts are difficult to quantify. It is estimated that 32 million tonnes of yield are lost to leaf rust annually. 20 million tonnes are lost to stripe rust and 9 million tonnes to stem rust (Hafeez et al. 2021). While stem rust causes the lowest yield losses globally, its impact is much more destructive in the tropics than elsewhere. It is estimated that more than 5% of wheat yield is lost to it annually in Sub-Saharan Africa (Savary et al. 2019).

Establishment of disease is dependent on the availability of inoculum. The spores of *Pgt* can be easily transferred by wind currents over very long distances and can establish new foci every season even in places where the pathogen cannot overwinter (Brown & Hovmøller 2002). The spread of inoculum can be intercontinental (Meyer et al. 2017; Visser et al. 2019), and the spores travel in wind currents thousands of meters above ground. In some areas the migration of inoculum occurs seasonally from areas where the fungus overwinters to places where a crop is grown seasonally. An area with earlier growing season can serve as a source of inoculum for areas where crops are grown later in the year. In United States this is referred to as *Puccinia* pathway (Aylor 2003) and similar patterns have been projected on other continents (Radici et al. 2022). Additionally, spores can be also transmitted by human activity, transferred over long distances on clothing or goods. This was probably the mechanism behind introduction of stripe rust to the Australian continent in the 1970's (Wellings et al. 1987).

Another driver in disease impact is the specificity of strains and host-pathogen interaction. A strain that devastates wheat crops in one area may not be able to pose much threat in another place in the world due to either different climatic conditions or incompatibility with the local cultivars. On the other hand, a local strain that evolves in habitat where it does not pose a threat to agriculture might

migrate to another continent and have an impact there. Therefore, stem rust epidemiology must be studied together with biogeography and agroecology of both pathogen and its hosts.

2.5.1. Impact in tropics and subtropics

Fungal diseases on crops generally tend to cause higher yield losses in the tropics and in developing countries. This has two main reasons: Firstly, higher temperature and humidity provide more suitable conditions for fungi and the lack of cold winter season means there is no need for overwintering in the life cycle. Secondly, in developing countries fungicides to control the diseases may be less readily available to the farmers.

Stem rust is the most important wheat rust disease in tropics as it is better suited to hot temperatures (Roelfs et al. 1992). It is especially important in wheat-growing regions of Ethiopia, Kenya, and Uganda. Ethiopia is the largest wheat grower in sub-Saharan Africa and the production is continuously increasing (Tadesse et al. 2022). Newly virulent races occur there regularly and challenge local wheat production. In 1993-1994, an epidemic was caused in Ethiopia due to a new strain virulent on then widely planted cultivar Enkoy. Yield losses on Enkoy were 65 – 100% (Dubin & Brennan 2009). In 1998, a new race of *Pgt* was detected in Uganda (Pretorius et al. 2000). This race was virulent to then widely utilized resistance gene *Sr31* and was named Ug99. Its virulence was described as TTKS according to international nomenclature (Roelfs & Martens 1988). Later, descendants of the original Ug99 race appeared with additional virulence such as virulence to gene *Sr24* (Jin et al. 2008), *Sr36* (Jin et al. 2009), and *SrTmp* (Patpour et al. 2016). Races in the Ug99 group then dominated Ethiopian and Kenyan wheat-growing areas and spread quickly both to the north into Sudan, Egypt, Yemen, and Iran as well as to the south into South Africa (Singh et al. 2015). There is now at least 13 recorded races in the lineage (Fetch et al. 2016) and the strains were placed in a genetic group I according to SSR genotyping (Olivera et al. 2015). The races from this genetic group that later appeared in countries including Kenya and South Africa include TTKSK, TTKST, TTTSK, TTHST, TTKTK, TTKTT, TTKSF, TTKSP, and PTKST (Szabo et al. 2022). This diversity demostrates the great potential to differentiate when strains are successful. Race TTKTT in particular is of great concern and appeared recently in collections in Ethiopia in 2018 (Hei et al. 2020) and Iraq in 2019 (Nazari et al. 2021).

In 2013 and 2014, another outbreak of stem rust was recorded in Ethiopia, widely affecting a cultivar Digalu (Olivera et al. 2015). The race TKTTF responsible has since been called the *Digalu race* and it belongs to genetic groups IV-A and IV-B according to SSR genotyping (Olivera et al. 2015). Other races detected in Ethiopia in the years of the epidemics were TRTTF and RRTTF, belonging to genetic group III-A (Olivera et al. 2015; Szabo et al. 2022). Races recorded in this outbreak have successfully spread far outside the affected region and are now established in Africa

and outside. In the latest reports from Ethiopia, races TKKTF and TTTTF seem to be most prominent, followed by TTKTF, TTKTT, TTRTF, TKPTF and TKKTT (Gutu et al. 2022).

The wheat breeding for rust resistance in the tropics was driven by necessity ever since wheat was started to be grown in Kenya and Ethiopia at the beginning of 20th century. Severe devastation of wheat fields in 1906-1917 put rust resistance in the forefront of breeding programs. Breeding materials had to be sourced from other continents. An international cooperation of plant pathologists and breeders was necessary to produce resistant wheat lines to prevent reoccurring epidemics. It was mostly two organizations that provided materials to the east-African programmes – International Maize and Wheat Improvement Center (CIMMYT), and International Center for Agricultural Research in the Dry Areas (ICARDA).

2.5.2. Impact in Czech Republic and the European context

Out of the three wheat rusts, leaf rust is the most common in Czech Republic. Leaf rust occurrence has inspired breeding and research since 1960 when first cultivars with specific genes of resistance were introduced. Monitoring of pathogen races has been done since 1960s (Bartoš 2010). Historically, minor stripe rust epidemics have also been recorded in Czech Republic in 1926 and 1961, with local yield-losses of 30-50% (Zadoks 1967). A significant spread of stripe rust was recorded in the 1999 and subsequent years where virulent races were spreading from Western Europe (Bartoš 2010). Even though stripe rust is generally more frequent in countries with maritime climate such as Germany, Denmark, or Great Britain, it can also cause local outbreaks in continental Europe.

In Czech Republic, stem rust generally occurs less frequently than leaf rust. It is considered better adapted to higher temperatures typical for tropical wheat growing regions (Roelfs et al. 1992). Czech Republic is in the centre of Europe and mostly falls within the continental agro-climate zone (Ceglar et al. 2019). *Pgt* is not known to overwinter in the region and the secondary host probably has no importance for epidemiology of the disease, as only grass and rye forms of *P. graminis* have been found to complete the sexual cycle on local barberry (Bartoš & Valkoun 1980). This means that wheat stem rust is probably exodemic in Czech Republic, the primary inoculum must travel on wind currents from the southern regions of Europe and establish disease on susceptible cultivars at the beginning of each growing season to have any impact on grain production. The primary inoculum source might be Greece or Turkey, from where the spores migrate north annually following the east-European tract (Zadoks 1967). Historically, outbreaks causing yield losses were recorded in 1930s and 1940s, followed by mild epidemics in 1958 and 1962 (Zadoks 1967). Collections and studies of pathogen races in former Czechoslovakia began in year 1962. Minor epidemic was recorded in 1972 when *Pgt* population overcame resistance gene *Sr5* (Bartoš 2010).

Genes *Sr31* and *SrTmp* remained effective at that time. After 1972 stem rust has lost importance, occurred rarely, and populations were not monitored. In recent years, however, stem rust has been reappearing and new *Pgt* samples have been collected from fields and stored in the Collection of Biotrophic Fungi of the Crop Research Institute in Prague (more information about the collection available at https://www.vurv.cz/culturecollection/).

The recent increase of sample numbers corresponds with situation elsewhere on the continent, as stem rust is regaining importance all around. Increased amounts of samples have been collected in recent years (Patpour et al. 2022a), even in locations where stem rust had not been present for decades. The prevailing strains belong to genetic groups IV-B, IV-F, and III-B, all probably incursions from outside of the continent. Clade IV-B strains caused epidemics in Ethiopia in years 2013-2015 (Olivera et al. 2015) before being detected in Europe. Race TKTTF typical for this genetic group has now been sampled as far north as Sweden (Patpour et al. 2022b) and Ireland (Tsushima et al. 2022). Subclades of clade IV were responsible for an outbreak of stem rust in Germany in 2013, where several races were sampled. Those included TKTTF, TKPTF, TKKTF, TKKTP, and PKPTF (Olivera Firpo et al. 2017).

Clade III-B with its typical race TTRTF (often termed *Sicily race*) was responsible for an outbreak in Sicily in 2016 (Bhattacharya 2017). Since then, it was detected much further north in Sweden (Patpour et al. 2022b), among other locations in Europe. After 2016 this race was also detected outside of Europe, in Ethiopia in 2017 (Szabo et al. 2022), Eritrea in 2016, and Iran in 2019 (Patpour et al. 2020). Before the outbreak in Sicily, the *Sicily race* was sampled only once, in Caucasian region in Georgia, which could potentially be its original source (Olivera et al. 2019). This region is known for its barberry population and conductive environment for sexual propagation of the pathogen. Races with novel virulence spectra can emerge from such environment. A map in Figure 3 aggregates findings from six recent studies (Olivera Firpo et al. 2017; Lewis et al. 2018; Tsushima et al. 2022; Patpour et al. 2022a, 2022b; Rodriguez-Algaba et al. 2022) and includes approximate seasonal movements of inoculum from south to north (Zadoks 1967; Radici et al. 2022).

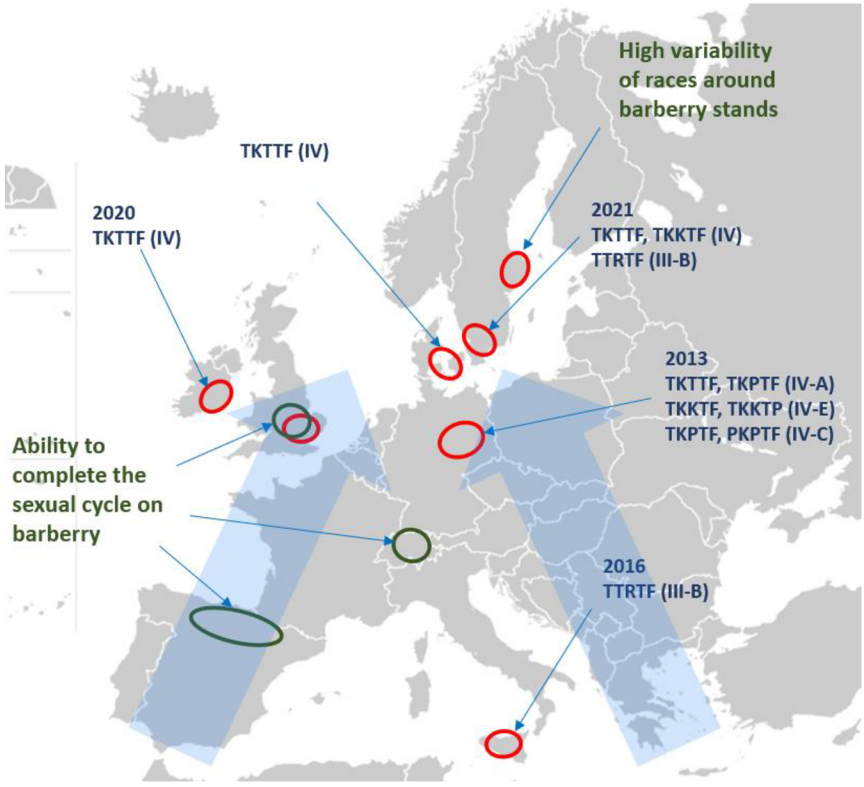

Figure 3: Map of Europe with recent stem rust occurences of interest. Red circles show locations of new pathogen races appearing, green circles show areas, where ability to complete the sexual cycle was recently proven with samples being able to reinfect wheat. Arrows show west-European and east-European pathways of seasonal spore spread from south to north (references in text).

Ug99 races that caused much concern because of the devastating impact in tropics were not confirmed in Europe and we can now assume that this genetic group has not adapted to the mild climate. This is in contrast with the *Digalu race* which first caused damage in Africa and is now impacting agriculture in Europe. Ug99 incursion would overcome the common resistance gene *Sr31* which is present in European wheat and remains effective for the time being. In Europe, *Sr31* virulence was previously only recorded in Spain in an isolate unrelated to Ug99 in a location with high genetic variability around barberry bushes (Olivera et al. 2022), but it was limited to that one isolate.

The impact of stem rust on yield loss in Czech Republic is very difficult to estimate. It is clear that in today's time most of the losses are prevented with the repeated use of fungicides throughout the season. The only quantitative data to be found in literature are estimations of losses during historical outbreaks in Czechoslovakia in 1932-1941, ranging between 40 000 to 275 000 tons of grain per each year when stem rust was present (Zadoks 1967). In the 1960's, experiments were done to assess the negative effect of stem rust on thousand grain weight (TGW) in Czechoslovakia (Bartoš & Šebesta 1966, 1968). These studies were undertaken in field conditions with artificial inoculations of a spreader, which is a susceptible cultivar sown between the tested lines to promote

disease spread in the trial. The spreader was inoculated with urediospores in May. They show that stem rust causes most damage when the plants are inoculated in the flowering stage and the impact is lower in later stages. 80% decrease of TGW was demonstrated in the most susceptible lines untreated with fungicides (Bartoš & Šebesta 1966). The decrease of TGW varied greatly between the years when the experiments were done, which was caused by varying weather conditions. However, even in year 1964, when the conditions were not optimal for stem rust spread and development, the average decrease of TGW in untreated winter wheats was 24 % (Bartoš & Šebesta 1968). This shows that weather itself is not as limiting for the disease impact in Czech Republic as is the timing of first natural infections. In natural conditions, primary inoculum is not available in May when these trials were inoculated. The main influx of spores in this altitude is to be expected later in summer months (Radici et al. 2022). Recent survey assessing worldwide crop loss to diseases and pests finds impact of stem rust in North-West Europe negligible and does not include Central or Eastern Europe as a study area (Savary et al. 2019).

Since impact of stem rust greatly depends on climatic conditions, climate change could be one of the driving factors behind its increased incidence in Europe. Data on incidence of multiple fungal pathogens throughout several decades were collected to fit a model which predicts that in the northern hemisphere, fungal crop pathogens shift 7.61 ± 2.41 km towards the pole each year (Bebber et al. 2013). In case of stem rust, of course, this would not manifest continuously but rather in increased incidence of local outbreaks. Increased temperatures in Europe due to climate change could increase the sporulation rate of stem rust. On the other hand, the drier conditions which are also projected could decrease the spore ability to germinate, since they require high humidity for that (Prank et al. 2019). And while climate change could limit the intercontinental spore transport from Africa to Europe, it is also projected to expand the area where the pathogen is able to overwinter. This might include Czech Republic (Prank et al. 2019).

All three wheat rusts are subject to study and breeding in Czech Republic. Farmers send samples of diseased leaves to Crop Research Institute in Prague where races are evaluated for virulence and increased and then stored in the carefully curated Collection of Biotrophic Fungi. Scientific articles on current state of local rust populations and resistance in local wheat are published periodically in international journals (Bartoš 1980; Hanzalová et al. 2012, 2020, 2021; Hanzalová & Bartoš 2014). The Central Institute of Testing and Supervising in Agriculture (ÚKZÚZ) tests newly registered wheat cultivars for resistance to the three rusts, and commercial breeding companies take resistance to rusts into consideration when breeding new varieties.

2.6. Genetics of resistance

There are currently more than 80 stem rust resistance genes described in wheat and over 60 catalogued with a number from *Sr1* (although *Sr1* is now an abandoned designation) to *Sr61*, which is the most recent in time of writing this thesis. The genes can be categorized based on several criteria. Most commonly they are divided into all-stage resistance vs. adult plant resistance (APR) genes based on the growth stage during which they are effective. All-stage resistance can be detected in seedlings which makes selection for them easier. Based on its genetic basis the resistance is either qualitative or quantitative. Resistance can be inherited monogenically or oligo/polygenically. Another important factor is the race-specificity – some genes are race-specific while others are effective against all known races of the pathogen.

Even though quantitatively inherited APR is usually non-race-specific, this is not a general rule, as was demonstrated on quantitative trait loci (QTLs) that lost their efficacy against novel *Pst* races (Sørensen et al. 2014). While categorizing genes makes matters easier, it is also necessary to also address them individually. Following chapters will address some common groups of genes and then individual genes with significant relevance will be addressed.

2.6.1. Major resistance genes

Major resistance (R) genes are effective only against some races of the pathogen and confer a qualitatively measured resistance. The variability in virulence of isolates towards these genes can be explained by the gene-for-gene concept (Flor 1971); for every gene that confers resistant reaction in the plant, there is a corresponding gene in the pathogen that conditions avirulence. This hypothesis was based on Mendelian inheritance tests performed with flax rust pathogen *Melampsora lini* and its host *Linum usitatissimum*, however it explains relationships between many other pathogens and their hosts.

During an incompatible (avirulent) reaction, the plant can detect a pathogen's secreted effector protein by its own receptor protein, which triggers the resistant reaction (hypersensitive reaction, HR). The pathogen's protein is encoded by an *Avr* gene while the receptor protein of the host is encoded by the *R* gene. Naturally this interaction is highly specific and once either the pathogen or the host lose the ability to express their *Avr* or *R* genes, it can no longer happen. If the pathogen does not express the *Avr* gene, the plant does not detect being infected. If the pathogen expresses the gene but the plant does not produce the corresponding receptor, the defensive mechanism cannot be triggered either. This mechanism explains why deploying cultivars with a single major R gene in large-scale agricultural production cannot be a viable long term defence strategy. Once the pathogen mutates into a new race in which the *Avr* gene is no longer expressed, it can successfully

infect susceptible plants on which it propagates quickly. The structure of the encoded proteins was not known in times of Flor (reviewed here in chapter 2.6.3.) and the research relied on Mendelian inheritance experiments. Even so, it explained a crucial part of pathogen-host relationships: How a resistant cultivar suddenly becomes susceptible to its pathogen.

One significant example of cereal rusts overcoming host resistance is the emergence of *Pgt* race Ug99 in Uganda in 1998. Ug99 was a new race with a virulence to then widely used resistance gene *Sr31* (Pretorius et al. 2000). The *Sr31* virulence together with virulence to other genes such as *Sr38* allowed this race to cause significant damage and disperse across Africa and eventually the Middle East (Singh et al. 2011). The original race was described as TTKSK using the American nomenclature, but the abundance of susceptible hosts allowed it to propagate and create mutants with additional virulence, such TTKST with virulence to *Sr24* (Jin et al. 2008) or TTTSK with virulence to *Sr36* (Jin et al. 2009). Yellow rust gene *Yr9* is another historical example of a major resistance gene losing efficacy to newly virulent races of rust. The *Yr9*-virulent races were first recorded in the wheat-growing regions of Africa in the 1980s and then spread to Middle East and India (Wellings 2011). Furthermore, *Yr9*-virulent races caused outbreaks of yellow rust in China (Chen et al. 2009) and North America (Chen et al. 2002), where resistance was largely dependent on growing cultivars with this gene.

2.6.2. APR resistance genes

APR genes are a special case of resistance genes that become effective at later growth stages of the plant and are usually not race-specific. Instead of triggering a HR they slow down the growth of the pathogen inside the plant tissue. Even though they do not provide sufficient resistance individually, combining several of them together can achieve near immunity. Furthermore, such loci are often effective against multiple pathogens, and as such they attract attention of scientists and breeders. Three of the most studied genes *Yr18*, *Yr29*, and *Yr46* have a pleiotropic effect; they are effective against all three of the wheat rusts and powdery mildew. Their more complete designations are *Lr34/Yr18/Sr57/Pm38*, *Lr46/Yr29/Sr58/Pm39*, and *Lr67/Yr46/Sr55/Pm46*, respectively. A leaf-tip necrosis is a typical phenotypic trait that can be observed in lines harbouring these three genes. The ideal strategy to combat stem rust in the field is combining APR and R genes in cultivars to achieve a more durable resistance.

2.6.3. Resistance mechanisms

Resistance genes in plants encode proteins of different classes and functions and only a minority has been cloned and characterized. Universally, active resistance requires triggering of the resistant reaction via recognition of the pathogen attack. For this, receptors need to be present (Cook et al.

2015; Outram et al. 2022). There are two known distinct types of receptors in plants. First are the cell-surface pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs) and trigger the PAMP-triggered immunity (PTI). PTI is known as the first level of plant's defense because it relies on a general perception of a broad range of pathogens via their common conserved patterns (Fahima & Coaker 2023). The second type of receptor is intracellular and recognizes effector proteins (virulence factors) that pathogens emit inside the plant. Those receptors either directly or indirectly detect the effectors and trigger downstream processes known as effector-triggered immunity (ETI). The ETI is known as the second level of plant's immunity and is significantly more specific in recognizing pathogens. ETI is accepted as the explanation of molecular mechanisms behind the gene-for-gene relationship (chapter 2.6.1.), however, current knowledge already reveals more complexity on top of the classic binary view of the pathogen-host interaction. Since many receptors require co-receptors or additional proteins to function and they trigger an array of common downstream components, they in fact form a complex signaling network (Kamoun et al. 2018). Additionally, even though PTI and ETI require different receptors, they often trigger common downstream mechanisms, suggesting these two layers of defense interact extensively (Yuan et al. 2021).

Most of ETI receptors in plants fall in the family of nucleotide-binding leucine-rich repeat receptors (NLRs) (Maruta et al. 2022). Plants developed a wide array of such receptors. Most of the rust resistance genes that have been cloned fall in this category. NLRs have a typical structure consisting of three domains: (i) a variable N-terminal domain, (ii) a central nucleotide-binding domain, and (iii) C-terminal leucine-rich repeat domain (LRR). In plants, the central nucleotide-binding domain belongs to a NB-ARC domain class and acts as a molecular switch with an inactive and active form. It is the LRR domain that binds to the pathogen effector and activates the central domain. When the central domain is activated, the NLRs form a signaling assembly known as a resistosome (Wang et al. 2019). The N-terminal domains are then responsible for downstream signaling. In plants, several N-terminal domains have been described in NLRs. Most importantly, some of NLRs include the Toll interleukin-1 receptor (TIR) and some include a coiled-coil (CC) motif. Several plant resistance genes that encode each of those NLR families have been cloned. In case of stem rust specifically, out of the 17 resistance genes that have been cloned, 12 encode NLRs, all with the CC motif (Yu et al. 2023). Those are *Sr13* (Zhang et al. 2017), *Sr21* (Chen et al. 2018b), *Sr22* (Steuernagel et al. 2016), *Sr26* (Zhang et al. 2021), *Sr27* (Upadhyaya et al. 2021), *Sr33* (Periyannan et al. 2013), *Sr35* (Saintenac et al. 2013), *Sr45* (Steuernagel et al. 2016), *Sr46* (Arora et al. 2019), *Sr50* (Mago et al. 2015), *Sr61* (Zhang et al. 2021), and *SrTA1662* (Arora et al. 2019).

Two stem resistance genes *Sr60* and *Sr62* encode proteins with two tandem kinases (Chen et al. 2020; Yu et al. 2022). The function of the kinases is not known. However, it is speculated that those tandem kinase resistance genes require associated NLRs to function (Yu et al. 2022). Recently, *Sr43* was cloned and shown to encode a kinase with two domains of unknown functions (Yu et al. 2023). The two remaining cloned genes *Sr55* and *Sr57* will be discussed in their dedicated chapters.

2.6.4. Molecular markers

To be able to work with resistance genes at all, breeders and researchers need an information on their presence in breeding materials, parental lines, and cultivars. Because inoculation experiments and field trials can take a lot of time, especially if information on several genes is needed, it is helpful to use molecular markers to confirm the presence of genes quickly. This approach is called marker-assisted selection (MAS) when used in breeding (Gupta et al. 1999; Paux et al. 2012). There are several types of markers that have been used to determine presence of resistance genes. The commonly used types of PCR-based markers are listed in Table 3 and include simple sequence repeats (SSR), sequence-tagged site (STS), sequence-characterized amplified region (SCAR), and cleaved amplified polymorphic sequences (CAPS). Now, Kompetitive allele-specific PCR (KASP) markers are becoming popular (Rasheed et al. 2016; Kaur et al. 2020). All of those are based on polymerase chain reaction (PCR) and reading of amplification levels. In addition, CAPS requires application of a restriction enzyme to cleave the amplified product and to make a distinction between single-nucleotide polymorphisms (SNP). KASP markers utilize fluorescently tagged primers recognize SNPs, therefore a quantitative PCR machine or a microplate reader is needed to evaluate the results. TaqMan probes require a qPCR machine to read the fluorescence after each cycle.

Table 3: Types of PCR-based molecular markers used in wheat marker-assisted breeding and research. Adapted from Maccaferri et al. (2022).

Technology	Requirements	Short description
STS (Sequence-tagged site)	PCR + electrophoresis	Short DNA sequence that is specifically amplified if present
SSR (Simple sequence repeats) and ISSR (inter-simple sequence repeats)	PCR + electrophoresis (polyacrylamide)	Polymorphisms are identified by primers flanking a region of short repeating units as amplicons have different lengths
SCAR (Sequence-characterized amplified region)	PCR + electrophoresis	Primers designed from sequences of RAPD fragments (Random amplified polymorphic DNA)
AFLP (Amlified fragment length polymorphisms)	PCR + electrophoresis (polyacrylamide)	Selective amplification of restriction fragments of variable lengths.
CAPS (Cleaved amplified polymorphic sequences)	PCR + enzyme digestion + electrophoresis	A region containing a SNP is amplified, then a digestion enzyme is used to identify the polymorphism
Real-Time hydrolysis probes (TaqMan®)	Real-time PCR	Quantitative PCR with increased specificity. Fluorescence from a reporter dye is measured after every cycle.
KASP (Kompetitive allele-specific PCR)	PCR + end-point fluorescence measurement (on either qPCR machine or a plate reader)	Two allele specific forward primers are tagged with a distinct fluorescent dye, then fluorescence is measured and separates the two distinct alleles. This can be a SNP, indel, or multiple polymorphisms.

2.6.5. Breeding for rust resistance

In breeding for new commercial cultivars, resistance to disease is only one of the objectives and stem rust is only one of the diseases that have potential to reduce yields. A line cultivar with a sufficient and durable resistance is the desired output of breeding programs. Some classical breeding methods can be used to increase resistance. Those are backcross breeding, recurrent selection, and multi-stage selection.

Firstly, backcross (BC) breeding is a method to introduce a single target gene from donor into a recipient line of choice. In this method, the recipient line is a recurring parent used for backcrossing of the new generation. With each backcrossing step, the recurrent parent genome enriches and progeny that include the desired gene is selected. This is repeated until as much as possible of the genome comes from the recipient line and the gene from donor is successfully introgressed. The number of generations needed differs based on the genetic difference between donor and recipient. In practice, the recurrent parent is often changed between generations (Miedaner 2016). After the last backcrossing step, the selected progeny needs to be selfed to produce homozygous lines (Miedaner 2016; Khan et al. 2020). BC breeding serves as a tool to introduce genes from non-elite cultivars or from wild relatives. Such method can take around ten years to complete to introduce a single gene, even though the resistance conferred by single gene is usually not durable in case of stem rust. Molecular markers can be used to speed up the selection process (Miedaner & Korzun 2012).

Recurrent selection is the second traditional breeding method to increase resistance to stem rust. This method is designed to increase frequency of alleles for quantitatively inherited resistance, while also maintaining the genetic diversity. This is done by interbreeding of selected progeny in repeated steps. The goal of this method is increasing the resistance in the whole breeding population, the so-called horizontal resistance. Success depends on population size and selection intensity. It can be hindered by negative association of resistance with yield traits (Miedaner 2016).

In practice, since there are many traits to select for, a multi-stage selection is mostly used in breeding programs. This allows to screen for resistance to different pathogens in each step, even multiple ones per generation. Often, several locations are used for disease resistance testing. To speed up the selection process, MAS for major genes or QTLs is possible throughout the breeding process. Double haploid (DH) lines that are pure homozygotes can be generated by in-vivo parthenogenesis and subsequent polyploidization. This means that several generations of selfing are not needed to produce a pure line and time is saved.

Among more modern breeding methods, genomic selection (GS) (Meuwissen et al. 2001) is available for rust resistance breeding (Rutkoski et al. 2011, 2014, 2015). Genomic selection uses genome-wide markers to capture total additive genetic variance. In this method, the effect sizes of all used markers are predicted using a statistical model. First, a training population is required. This is a panel of wheat lines for which both genotypic and phenotypic data are produced. From those datasets the prediction model is fitted to predict genomic estimated breeding values (GEBVs) across the genomes. The marker set that was applied for the training population can then be applied in a panel of selection candidates and selection can be done without phenotyping, only by predicting the GEBVs. The main advantage of GS is the high resolution, as even low-effect QTLs are considered in the selection process. GS has been successfully used for stem rust APR in a CIMMYT wheat panel (Rutkoski et al. 2014), for yellow rust and stem rust resistance in five panels of CIMMYT wheats (Ornella et al. 2012), and for all three wheat rusts in a diverse panel of landraces (Daetwyler et al. 2014).

With the rise of gene engineering techniques and especially gene editing method CRISPR (acronym for clustered regularly interspaced short palindromic repeats), wheat breeding is advancing in this direction rapidly, as was reviewed several times in recent years (Borisjuk et al. 2019; Wang et al. 2020; Li et al. 2021). In case of stem rust resistance breeding, perhaps the most significant use of gene engineering was the introgression of five resistance genes in a single construct (Luo et al. 2021). In this experiment, *Sr22*, *Sr35*, *Sr45*, *Sr50* and *Sr55* including some of their regulatory elements were all transferred into a cultivar Fielder using *Agrobacterium*-mediated transformation. It was then proven with phenotypic tests that at least four of those genes are effective in the recipient

line independently. This approach significantly simplifies gene pyramiding, which is very tedious if only classical breeding methods are used. Using five major genes at once provides near complete resistance that is difficult to break down by new races. While this is a promising technological development, lines produced through transgenesis are GMOs and legislation in Europe and elsewhere hinders their use in agriculture. In European Union, GMO regulations have been considered especially strict (Davison & Ammann 2017).

2.7. Selected stem rust resistance genes

2.7.1. Sr2

Resistance gene *Sr2* was transferred to common wheat cultivar Marquis from emmer wheat (*Triticum dicoccum* Schronk) cultivar Yaroslav, creating a new line of wheat named Hope (McFadden 1930). The major use of this gene came with cultivar Newthatch and the cross FKN (=Frontana/Kenya 581 /Newthatch) from which it has spread into much of CIMMYT breeding germplasm (Van Ginkel & Rajaram 1993). *Sr2* is an APR gene that is partially effective against all *Pgt* races. However, it is recommended to be combined with other resistance genes as it is not sufficient on its own when the disease pressure is heavy (Singh et al. 2011). *Sr2* has been widely used in United States and in CIMMYT breeding program cultivars (Huerta-Espino et al. 2020). It is not known whether it is present in cultivars registered in Czech Republic.

Sr2 is mapped to chromosome 3BS and is associated to a leaf rust resistance gene *Lr27*, a powdery mildew resistance gene, and a pseudo black chaff (*PBC*) gene (Kota et al. 2006; Mago et al. 2011a). *PBC* is a trait causing a distinct pigmentation of stems and glumes that has been used as a visual aid for selection of lines carrying *Sr2*. Several molecular markers have been suggested to aid breeders in selection for the gene (Spielmeyer et al. 2003; Hayden et al. 2004; McNeil et al. 2008; Mago et al. 2011b), out of which a CAPS marker *csSr2* currently appears to be the most reliable (Bernardo et al. 2013).

2.7.2. Sr6

Origin of the *Sr6* resistance gene can be tracked back to Red Egyptian, Kenya 58, and related African cultivars (Knott 1957; Green et al. 1960). It has been widely studied in the past especially with regards to its physiology because its effectiveness is dependent on temperature and light. *Sr6* has been frequently utilized in commercial varieties. Zhang et al. (2014) reported a 12% frequency of *Sr6* in United States winter wheat. To my knowledge, *Sr6* has not been reported in any Czech cultivars that are currently grown. However, it was reported in cultivar Košútka registered in Czechoslovakia in 1981.

Sr6 is mapped to chromosome 2DS. Tsilo et al. (2009) showed that SSR markers *Xcfd43* and *Xwmc453* are predictive of *Sr6* presence, and the former proved to be more reliable and easier to score (Ejaz et al. 2012; Mourad et al. 2018).

2.7.3. Sr8a

Sr8a is located on chromosome arm 6AS and is a common gene that is present in such cultivars as Red Egyptian, Frontana, Mentana, and others. It is probably present worldwide. In Europe, its presence was postulated in Agron, Asta, Barleta, Viginta, and Boka (Pathan & Park 2007) and spring wheats Taifun and KWS Scirroco (Flath et al. 2018). It is present in the North American differential set, in isogenic line ISr8-Ra (Roelfs & Martens 1988).

Its effect in the field is not very distinctive and even avirulent isolates can cause strong disease symptoms under high infection pressure. Nevertheless, it may still play a role in stem rust epidemiology since the widespread novel races TRTTF and RRTTF are avirulent to it. Two SNP markers wsnp_Ku_c39334_47795461 and wsnp_Ra_c3996_7334169 were proposed to detect the gene (Guerrero-Chavez et al. 2015). Another team of researchers (Edae et al. 2018) used a linked KASP marker kwh54 (Excalibur_c12085_276), which was mapped 2.2 cM away from *Sr11* in another experiment (Hiebert et al. 2017).

2.7.4. Sr11

Sr11 is located on a 6BL chromosome arm and originally comes from a durum wheat cultivar Gaza (McIntosh et al. 1995). *Sr11* is part of differential sets including the one used for North American nomenclature (Roelfs & Martens 1988). It is presumably very common worldwide, since it was present in many old CIMMYT lines, and it was also very common in Australia where virulent isolates completely overcame this gene to the point when *Sr11* lost any importance there. The virulence frequency might be lower on other continents since it varies greatly. It was detected in 21% of spring wheats in a selection from North America (Edae et al. 2018). In Europe, *Sr11* was postulated in Astron, Blava, Boval, Contra, Kontrast, Monopol, Yalta, (Pathan & Park 2007) and Flevina (Bartoš et al. 1970). Based on experiments with stem rust races in former Czechoslovakia in 1966-1971, *Sr11* was described as broadly effective, with only few isolates showing intermediate response to it (Bartoš 1972). During race phenotyping of local isolates in years 1972 and 1973, no susceptible reactions to *Sr11* were found (Bartoš 1975a). Same results were obtained in samples collected in 1974-1976 (Bartoš & Hladká 1978). One virulent isolate was collected in 1977 (Bartoš et al. 1982). Even though virulent races are now common in populations around the world, *Sr11* might still play a role in epidemiology of stem rust since the novel, widespread race TKTTF is avirulent to it.

Nirmala (2016) proposed linked KASP markers KASP_6BL_IWB73072 and KASP_6BL_IWB46893 to detect the presence of *Sr11* in germplasm.

2.7.5. Sr22

A stem rust resistance gene *Sr22* is located on wheat chromosome arm 7AL and it originally comes from *Triticum boeoticum* and *Triticum monococcum* (Gerechter-Amitai et al. 1971; Kerber & Dyck 1973). It is a race-specific and temperature dependent gene which is more effective in lower temperatures (McIntosh et al. 1995). *Sr22* has been reported in wheat cultivar Schonburgk registered in Australia (McIntosh et al. 1995), but its wider use in worldwide breeding has been hindered by a significant linkage drag. More recently, wheat lines with shorter introgressions carrying the *Sr22* segment have been introduced (Olson et al. 2010a).

Markers *Cfa2123*, *wmc633*, and *csIH81-BM*/*csIH81-AG* have been used to detect *Sr22* in the past (Khan et al. 2005; Olson et al. 2010a; Periyannan et al. 2011). Using winter wheats from United States, Bernardo et al. (2013) demonstrated that the marker pair *csIH81-BM*/*csIH81-AG* can be used reliably. Marker *csIH81-BM* amplifies the resistant 257 bp allele whereas *csIH81-AG* amplifies the susceptible 385 bp allele. This pair of markers can be used together in one PCR reaction.

2.7.6. Sr24

Sr24 is completely linked to a leaf rust resistance gene *Lr24* and is derived from *Thinopyrum ponticum*. The segment containing *Sr24*/*Lr24* can be either located on chromosome arm 3DL as it is in wheat cultivar Agent (McIntosh et al. 1977) or on chromosome arm 1BS as in cultivar Amigo (The et al. 1991). *Sr24* is present in wheat germplasm grown around the globe including temperate Europe. It was postulated in eight German cultivars (Flath et al. 2018) including Elixer and Gordian which are also registered in Czech Republic and present in our study. In our previous study focused on leaf rust resistance, *Sr24*/*Lr24* was detected in three Czech cultivars out of 19 tested (Hanzalová et al. 2020).

It is possible to detect the *Sr24*/*Lr24* segment using markers Sr24#12, Sr24#50, or Xbarc71, all located distally on 3DL (Mago et al. 2005a; Liu et al. 2014). Gupta et al. (Gupta et al. 2006a) introduced a SCAR marker SCS1326-615 which proved to detect *Lr24* reliably and irrespective of which chromosome it was located on (Uhrin et al. 2008).

2.7.7. Sr25

A translocation from *Thinopyrum ponticum* was transferred onto common wheat chromosome arm 7DL by irradiation in the 1960's (Sarma & Knott 1966), resulting in a line named Agatha. The translocated segment contained the leaf rust resistance gene *Lr19*, stem rust resistance gene *Sr25*,

but also the undesirable yellow flour pigment gene Y. Because the initial translocation replaced almost the entire chromosome arm 7DL and the entire block was not expected to recombine with wheat chromosomes, there have been efforts to shorten the segment with desirable genes to make it more suitable for further breeding (Prins et al. 1997; Marais et al. 2001).

More recently, wheat lines with the *Lr19*/*Sr25 Th. ponticum* segment were recombined with lines containing homologous segments from *Th. intermedium* with resistance to barley yellow dwarf virus (BYDV) (Ayala-Navarrete et al. 2007). Lines combining genes *Bdv2*/*Lr19* and *Lr19*/*Sr35* were designated as Pontin, some of them with the segment reduced to 20% of the length of the original translocation (Ayala-Navarrete et al. 2013).

Several molecular markers have been used to detect the translocated segment in wheat. Notably, marker *Gb* was developed to detect *Lr19* by amplifying a product of 130 bp (Prins et al. 2001). It has been used to detect *Sr25* regularly since (Zheng et al. 2014; Kielsmeier-Cook et al. 2015; Xu et al. 2017, 2018; Khan et al. 2017). Sequence tagged site (STS) marker *BF145935* was introduced later, amplifying several loci in most wheat lines (Liu et al. 2010). The product of 198 bp is diagnostic of *Th. ponticum* translocation. When compared, BF145935 was deemed preferable to Gb due to consistent results and easier scoring (Yu et al. 2009). A SCAR marker *SCS265-512* was also introduced to detect *Lr19* (Gupta et al. 2006b). When compared to Gb by Uhrin et al. (2008) it was considered preferable due to being easier to use. It is important to note that neither of the markers have been developed from the actual *Sr25* sequence and therefore they are only diagnostic of the *Th. ponticum* translocation. It is possible that when a shorter segment of the translocation is introduced in new wheat cultivars, the markers will produce false results. More specifically, markers closely linked to *Lr19* might lose the ability to detect *Sr25* if the genes are separated.

2.7.8. Sr31

Resistance gene *Sr31* comes from the short arm of rye (*Secale cereale* L.) chromosome 1B. It has been transferred to wheat together with genes conferring resistance to yellow rust, leaf rust, and powdery mildew: *Yr9*, *Lr26*, and *Pm8*, respectively. These four genes are closely linked but not the same locus, as they were successfully separated in mutation studies (Mago et al. 2005b). Still, in most commercial cultivars, they are present together. Most wheat cultivars with *Sr31* contain a 1BL.1RS translocation that comes from Petkus rye. Kavkaz and Aurora are considered the original wheats with the translocation, followed by hundreds of cultivars. 1BL.1RS translocation is perhaps the most successful wheat-alien translocation in wheat breeding and has been widely utilized on all five continents (Friebe et al. 1996; Rabinovich 1998). Alternatively, this gene was also introduced with 1B (R) substitutions or a 1AL/1RS translocation which is present in cultivar Amigo (Rabinovich 1998). This gene has been studied in former Czechoslovakia especially in relation to

cultivars Salzmünder Bartweizen and Kavkaz, both used extensively in breeding of Eastern European wheats (Bartoš & Bareš 1971; Bartoš et al. 1973a, 1973b). *Sr31* has been postulated in numerous wheat cultivars registered in Czech Republic in the 1980's and 1990's (Bartoš et al. 1994a, 2002).

It is possible to detect the transferred rye DNA segment using an STS marker developed by Mago et al. (2002) from a RFLP probe iag95. The marker is co-dominant and segregates with the segment derived from Petkus rye.

2.7.9. Sr36

Sr36 was introduced into wheat from a related species *Triticum timopheevii* Zhuk. in several distinct and unrelated introgressions. It is mapped to chromosome arm 2BS (Yu et al. 2014). This gene has been commonly utilized in United States, where Zhang et al. (Zhang et al. 2014) detected it in 9% winter wheat lines. In a panel of 179 Ethiopian spring wheat lines, *Sr36* was detected in 30% of the lines. In Europe it has been reported in Hungarian and Croatian wheat (Purnhauser et al. 2011). Frequency in contemporary Czech cultivars is not known, but it was present in a 1985 Czechoslovakian cultivar Agra.

Markers Xgwm319, Xwmc477, and Xstm773-2 were suggested for *Sr36* detection in wheat by Tsilo et al. (2008), later Olson et al. (2010b) demonstrated that Xwmc477 was the most reliable of the three. However, even Xwmc477 was shown to produce some false positive results within an association mapping study of United States winter wheat (Zhang et al. 2014). Other sources also suggest a KASP marker wMAS000015 associated with the gene (https://maswheat.ucdavis.edu/protocols/Sr36), or IWB26389, which showed identical results in a study on Ethiopian spring wheat panel (Muleta et al. 2017).

2.7.10. Sr38

Sr38 was identified on wheat chromosome arm 2AS together with a leaf rust resistance gene *Lr37* and a yellow rust resistance gene *Yr17* (Bariana & McIntosh 1993). The three genes are tightly linked and were inherited as a cluster from *Triticum ventricosum* (Tausch) Ces. (Bariana & McIntosh 1994). This cluster has been used regularly in breeding for combined resistance and as a result it is quite common in wheat grown around the world. For instance, in a study of local Chinese cultivars, it was detected in 9 out of 75 varieties (Xu et al. 2017). In a study of North American winter wheat, it was detected in 26 varieties out of 137 (Zhang et al. 2014). The frequency of *Sr38* seems to be even higher in Europe, where Flath et al. (2018) postulated it in 29 German cultivars of 97 tested. Among those, there are cultivars Elixer, Bernstein, Brilliant, Genius, and RGT Reform that are also registered in Czech Republic.

A pair of primers *VENTRIUP* and *LN2* was introduced for detection of *Lr37-Yr17-Sr38* cluster by Helguera et al. (Helguera et al. 2003) and has since been successfully used in studies monitoring rust resistance (Bulos et al. 2006; Hanzalová et al. 2009; Ejaz et al. 2012; Cristina et al. 2015; Xu et al. 2018; Mutari et al. 2018).

2.7.11. SrTmp

SrTmp is named after a US winter wheat variety Triumph 64, in which it was identified. It was also found in other US winter wheat varieties, all presumably inheriting it from older eastern European lines (McVey & Hamilton 1985). *SrTmp* was mapped to a distal end of a chromosome arm 6DS (Lopez-Vera et al. 2014), where *SrCad* from variety Cadillac, and *Sr42* are also located (Hiebert et al. 2016). Because those three genes are located very close and might even share the same locus, their identification and differentiation by molecular markers is complicated.

2.7.12. Lr20 / Sr15 / Pm1

Sr15 was first described in wheat cultivars Norka and Thew (Watson & Luig 1966). *Sr15* is probably the same gene as *Lr20*, and therefore also confers resistance to leaf rust. It is closely linked to a powdery mildew resistance gene *Pm1*, but those two are separate genes (McIntosh et al. 1995). *Rlnn1* conferring resistance to a root lesion nematode was also shown to be closely linked with *Sr15*/*Lr20* (Jayatilake et al. 2013). This whole cluster of genes is located on chromosome arm 7AL and possibly occupies a rearranged segment of the chromosome which causes the low recombination rates between the individual genes. The effect of *Sr15* on avirulent isolates is dependent on low temperature, with resistance disappearing as temperatures go up. Therefore, it is possible to record susceptible reactions even between avirulent isolates and lines with *Sr15* in 22-25°C (Gao et al. 2019).

Sr15 is present in some older European wheat cultivars such as Maris Halberd, Timmo, Lona, Norka, Normandie, Sappo, Sicco (Pathan & Park 2007) and was recently found in Hungarian cultivar MV Zelma (Gao et al. 2019). It is also present in some Australian lines such as Angas, Aroona, Fedka (McIntosh et al. 1995). It is considered highly unreliable due to high levels of virulence on all continents and its dependency on low temperatures, however it can be helpful in combination with other genes and is effective against the important TTKSK race (Gao et al. 2019).

Several molecular markers have been used to confirm presence of *Sr15*, such as STS638, which was initially used to detect the *Lr20* and *Pm1* genes (Neu et al. 2011). More markers include wri4 (Jayatilake et al. 2013), and KASP_IWB30995 (Babiker et al. 2015). The three markers mentioned above almost always provide correct results (Gao et al. 2019).

2.7.13. Lr34 / Yr18 / Sr57 / Pm38

A slow-rusting APR gene on wheat chromosome 7D was designated *Lr34* by Dyck (1987). *Lr34* has a long history in breeding programs long before being named *Lr34* and is in fact present in wheat germplasm globally. Its pedigree can be traced back to South American wheats such as Frontana. *Lr34* is pleiotropic; it confers resistance to four diseases including powdery mildew caused by *Blumeria graminis*. *Lr34* was cloned and its sequence is known. It encodes an ATP-binding cassette (ABC) transporter (Krattinger et al. 2009). The slow-rusting and leaf-tip necrosis effects could not be separated, and gene expression studies imply that the mechanisms are directly connected. In a meta-QTL analysis (Soriano & Royo 2015), *Lr34* corresponds to locus MQTL34 on chromosome arm 7DL.

Lagudah et al. (2006) introduced a molecular marker csLV34 to detect *Lr34* resistance locus in marker-assisted selection. The marker has been used since. Recently, two KASP markers were introduced to differentiate between various alleles of the gene (Fang et al. 2020). Running both the Lr34-E11-KASP and Lr34-E22-KASP assays allows to detect a resistant allele that is present in Jagger and other winter wheats, or a different resistant allele that is present in spring wheat germplasm, such as cultivar Duster.

2.7.14. Lr46 / Yr29 / Sr58 / Pm39

Lr46 is a slow-rusting gene first identified in a cultivar Pavon 76, chromosome 1B (Singh et al. 1998). The gene was later mapped to the long arm of the chromosome and shown to be pleiotropic, conferring resistance against yellow and stem rusts and also powdery mildew (William et al. 2003; Lillemo et al. 2008; Kolmer et al. 2015). In this sense *Lr46* is like *Lr34*, which also provides resistance against multiple pathogens. In addition, both the genes are associated with leaf-tip necrosis trait. In a meta-QTL study of rust resistance APR genes done by Soriano and Royo (Soriano & Royo 2015), *Lr46* colocalizes with MQTL5.

2.7.15. Lr67 / Yr46 / Sr55 / Pm46

A new APR gene was identified in a wheat accession PI250413 in the 1970s (Dyck & Samborski 1979). This gene was later mapped to a chromosome arm 4DL and named *Lr67*.

It was shown to confer resistance against three rust diseases and powdery mildew, and to be closely linked to a leaf tip necrosis gene *Ltn3* (Herrera-Foessel et al. 2014a). *Lr67* was isolated and shown to encode a hexose transporter in two forms (Moore et al. 2015). Both the resistant form *LR67res* and the susceptible form *LR67sus* are upregulated during infection, but only the former confers resistance.

In a meta-QTL analysis of rust resistance APR genes undertaken by Soriano and Royo (2015), *Lr67* corresponds to MQTL20. Xcfd71-4D and Xcfd23-4D are markers that have been mapped close to *Lr67* and used to detect the gene in wheat germplasm (Hiebert et al. 2010). However, those two and additionally two even closer markers gwm4670 and gwm4866 have all been shown to produce false positives. The most reliable marker so far is a KASP marker csSNP856 that was produced by genotyping-by-sequencing approach (Forrest et al. 2014).

2.7.16. Lr68

Lr68 is an APR gene located on wheat chromosome 7BL, in proximity of a race-specific gene *Lr14b*. It was first identified in cultivar Parula but possibly comes from Brazilian cultivar Frontana from which it spread into other lines of the CIMMYT wheat breeding program (Herrera-Foessel et al. 2012). In a QTL meta-analysis analysis of slow-rusting leaf rust resistance genes done by Soriano & Royo (2015) a meta-QTL locus MQTL33 collocates with *Lr68* together with race-specific genes *Lr14* and *Lr19*. This locus corresponds to disease resistance QTLs found in other studies in cultivars Colosseo and Cresso (Maccaferri et al. 2008; Marone et al. 2013).

Two molecular markers were suggested for detection of *Lr68* in wheat germplasm. Cs7BLNLRR and csGS are positioned proximally and distanced 0.8 cM and 1.2 cM respectively (Herrera-Foessel et al. 2012). A KASP marker Lr68-2 based on a SNP CIMwMAS0056 is reported to be used in CIMMYT breeding (https://www.integratedbreeding.net/804/breeding-services/predictive-markers/genetic-information-leaf-rust-resistance-lr68).

2.8. Phenotyping rust resistance

2.8.1. Disease severity, infection types

The compatibility or incompatibility of the host-pathogen interaction, as well as disease severity, can be evaluated using several phenotyping methods. The simplest method to score disease severity in field trials is using a scale from 1 to 9, where 1 stands for minimal symptoms and 9 stands for most extensive disease symptoms. This scale is commonly used in cultivar trials and often supported by visual aid for the workers, such as in German national cutlivar trials (Bundessortenamt 2000). Description of such scale can also be found in Bariana et al. (2007). Due to its simplicity, results from other scoring systems are sometimes converted to this scale for statistical evaluation. It should be noted that some institutions use an inverted 1 – 9 scale where 1 stands for maximal disease symptoms and 9 implies highest resistance of the host plant, such as the Central Institute for Supervising and Testing in Agriculture of the Czech Republic (ÚKZÚZ, Horáková and Dvořáčková 2020). Another scale, used by United States Department of Agriculture (USDA), adds a 0 for no

symptoms and goes up to 9 for maximum severity (McNeal et al. 1971). There are conversion tables available between this and a descriptive scale or resistance classes (Miedaner 2016).

A more specific disease scoring approach aims at estimating the percentage of leaf and stem areas covered by rust pustules. This method uses the modified Cobb scale and a visual aid in form of diagrams is available to help with scoring (Peterson et al. 1948). The scale, same as the original Cobb scale, presumes that 100% disease severity equals 37% of area covered by pustules. The area of shaded portions (pustules) can be measured using a planimeter.

In field trials, six distinct host reaction categories (or resistance classes) can be discerned based on the symptoms (Table 4). Immune (I), resistant (R), moderately resistant (MR), moderately resistant-moderately susceptible (M), moderately susceptible (MS), and susceptible (S) (Ali & Hodson 2017).

Table 4: Host reaction categories and symptoms recorded during field scoring (Ali & Hodson 2017)

Abbreviation	Host reaction	Symptoms
I	Immune	No visible infection
R	Resistant	Necrotic areas with or without small pustules
MR	Moderately resistant	Small pustules surrounded by necrotic areas
M	Moderately resistant / moderately susceptible	Combination of both MR and MS
MS	Moderately susceptible	Medium-sized pustules, no necrosis, some chlorosis
S	Susceptible	Large pustules, no necrosis or chlorosis

When race typing rust pathogen samples or determining resistance genes in a cultivar, trials are done on seedlings in a greenhouse and in isolation to prevent contamination with undesired races. Since distinct resistance genes confer different mechanisms of resistance, each cultivar may react differently to each race of the pathogen. To describe distinct infection types, the Stakman scale is used (Stakman et al. 1962). This scale uses a combination of numbers and symbols (0, 1, 1-2, 2 – resistant, 3, 4 – susceptible, ; – chloroses, ++ and = indicate upper and lower limit of each type, ± indicates variation, X – mesothetic reaction).

2.8.2. Field trials, trap nurseries

To determine cultivars resistance in the field, disease trials are undertaken with a mixture of races that are currently present in the open landscape. Such trials do not need to be isolated from the influx of new inoculum from the atmosphere. They are aimed to mimic the actual, local conditions.

To get an early information about an influx of races and their virulence patterns, trap nurseries are grown. Trap nurseries consist of lines with known resistance gene composition, which may give early information on virulence of incoming races, before sample collections and greenhouse phenotyping can be made. This can be helpful in cases, where spread of inoculum needs to be monitored to give early warning (Ali & Hodson 2017).

2.8.3. Fluorescent microscopy

Distinct host-pathogen interactions can be also observed on a histological level, using a micro-phenotyping approach. It is possible to inspect the infection site under microscope with epifluorescence optics after staining the infected leaf segment (Moldenhauer et al. 2006). Different staining dyes can be used such as WGA-FITC that stains the chitin walls of mycelium (Zhang et al. 2017; Maree et al. 2020) or Fluorescent Brightener 28 (FB28, Calcofluor White M2R) that has been previously used in similar experiments (Ayliffe et al. 2011). Another possibility is using WGA Alexa Fluor 488 (Solanki et al. 2019) or Uvitex 2B (Dugyala et al. 2015). The fungal colony sizes can be then measured and any variabilities in the host reaction can be identified between the interactions (Sørensen et al. 2017b; Saleem et al. 2019). Figure 4 shows infection structures in a wheat leaf stained with WGA-FITC.

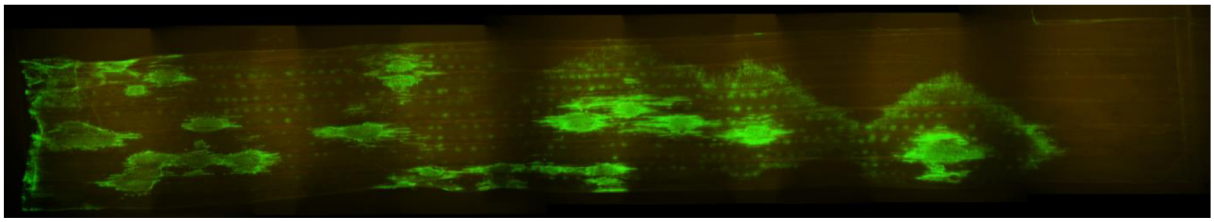

Figure 4: Fluorescent microscopy image of a susceptible wheat seedling leaf 10 days after inoculation with Puccinia graminis f. sp. tritici. Staining was done using wheat germ agglutinin conjugated with fluorescein. Image is composed from 7 individual microscopy photos stitched together to cover larger leaf area (image by author)

2.8.4. Fungal biomass quantification

Another method to study the host-pathogen interaction is to relatively quantify the level of infection by measuring fluorescence of stained infected plant tissue. WGA-FITC stain selectively binds to chitin in fungal structures in weighted and ground samples and samples with more severe infection show higher reads in fluorescent spectrometry reads (Ayliffe et al. 2013). This method was termed WAC assay. The advantage of this over visual assessment of disease severity is that it takes all

fungal tissue into account and not just ruptured pustules that are visible on the leaf surface with bare eye. Therefore, it is possible to reveal high level of colonization inside leaf tissue even if pustule production is slower.

Fungal biomass in infected plant tissues can also be relatively quantified by molecular methods using species-specific primers and qPCR. In such methods genomic DNA or mRNA is isolated from tested samples and genomic regions from the pathogen are quantified against genomic regions from the host. Different regions can be used for quantifying the pathogen such as β-tubulin gene from the distinct rust species (Maree et al. 2020; Maré et al. 2021). When using cDNA the reference gene from the host must be expressed with stability even during the infection process, however the stability of gene expression in wheat during infection differs according to the species of pathogen. Scholtz and Visser (Scholtz & Visser 2013) show that wheat ADP-ribosylation factor (ARF) gene is most suitable reference gene for *Pt* infection, 18S rRNA is most suitable reference for *Pgt* infection and cell division control (CDC) gene is most suitable for *Pst* infection (Table 5).

Table 5: Genomic regions used to quantify rust disease in wheat in the various pathogen-host interactions using qPCR

Pathogen/host	Gene	Primers	Reference
***Pt* (on wheat)**	β-tubulin	5'-CCTGAGTTGACCTCGCAAAT-3' 5'-CGAAACCTTACCACGGAAGTA-3'	(Maré et al. 2021)
***Pgt* (on wheat)**	β-tubulin	5'-CTCGATCGTGATGAGTGGGA-3' 5'-AGTGCAATCGAGGGAAAGGA-3'	
***Pst* (on wheat)**	β-tubulin	5'-CTCGGACGAAACCTTCTGTAT-3' 5'-CGTAGGTAGGTGTAGCCAATTT-3'	
Wheat (*Pt*-infected)	ADP-ribosylation factor (ARF)	5'-GCTCTCCAACAACATTGCCAAC-3' 5'-GCTTCTGCCTGTCACATACGC-3'	(Scholtz & Visser 2013)
Wheat (*Pgt*-infected)	18S ribosomal RNA	5'-GTGACGGGTGACGGAGAATT-3' 5'-GACACTAATGCGCCCGGTAT-3'	
Wheat (*Pst*-infected)	cell division control (CDC)	5'-CAAATACGCCATCAGGGAGAACATC-3' 5'-CGCTGCCGAAACCACGAGAC-3'	

2.8.5. Measuring aggressiveness

Some aspects of infection are not dependent on the host plant's resistance gene make-up but only differ quantitatively according to the pathogen's genetics. This is usually referred to as *pathogen aggressiveness*. Aggressiveness constitutes of several components that can be measured such as

infection efficiency, latent period, spore production rate, infection period and lesion size (Pariaud et al. 2009). The measuring of such infection components requires exact inoculation methods and repeated readings on the same infection site (Sørensen et al. 2017c). Aggressiveness in rust isolates is not often studied due to its technical demands.

2.9. Genomics of cereal rust pathogens

2.9.1. Reference genomes of rust pathogens

Since the emergence of next-generation sequencing, several reference genomes have been assembled for cereal rust pathogens. A haplotype-phased reference genomes are available for *Pgt* isolates Ug99 and *Pgt*21-0 (Li et al. 2019a). The most complete reference genomes for *Pst* yet are those of an Australian isolate *Pst*-104E covering 83.36 Mb in 156 contigs (Schwessinger et al. 2018), an American isolate 11-281 covering 84.75 Mb (Li et al. 2019b), and an isolate 93-210 covering 84.63 Mb (Xia et al. 2018). Those *Pst* genomes were aligned from long-read sequencing of DNA isolated from dried urediospores. Long-read sequencing was also used to compile the reference genome of *Pt*, race *Pt*104. It consists of 140.5Mb in 162 contigs (Wu et al. 2020). Later, a haplotype-phased reference genome was constructed for *Pt* (Wu et al. 2021).

2.9.2. RNA-seq methods to study rust pathogens

In the last ten years, several genetic studies have focused on using the transcriptome from either the collected fungal spores or from samples of infected plant tissues.

Hubbard et al. (2015) used RNA sequencing of pathogen-host samples directly from the field to study population shifts of *Pst* in Great Britain. They coined the term field pathogenomics. The main strength of the method is that the pathogen does not need to be propagated in greenhouse conditions to obtain enough spores, but instead the infected leaf which contains mycelium is collected and sequenced. The transfer from field to laboratory is enabled by storing the sample in RNAlater, a solution that stalls degradation of otherwise very fragile RNA. The rest of the work rests on bioinformatic software to separate the fungal and plant transcriptome and mine the data to establish genetic relations between the samples. The data evaluation process was standardized into several steps. First, it is necessary to filter and trim the raw sequences from Illumina. Next step is to align the reads to a reference genome of the pathogen. In case of rusts, about 30 % of the reads should align to the pathogen's reference genome and most of the rest should align to the wheat reference genome. If there is a large percentage of unaligned reads there might be an issue with contamination. The next step is SNP calling and then using the SNP sites to generate a phylogenetic tree and a defining the genetic groups using STRUCTURE software (Pritchard et al. 2000).

In the original study the authors described a change in the pathogen's population and increasing diversity in the samples. Furthermore, the fact that there was also plant RNA sequenced from the samples allowed them to study the transcriptome differences of the infected wheat varieties.

Since the method was established, it was also used to study other fungal pathogens of crops with samples directly from the field, such as *Fusarium* spp. (Fall et al. 2019) and *Magnaporthe oryzae* (Islam et al. 2016). Undeniably this is a very effective method and yields large amounts of data to mine from. However, the cost of RNA sequencing is still relatively high and requires trained personnel and modern equipment. Therefore, field pathogenomics is not yet a feasible option for regular large-scale pathogen monitoring and seems to be more fitting as a research tool.

Isolating and sequencing RNA allows for gene expression analyses. These can be done to study how gene expression profile changes in time or in different conditions, or to locate candidates for genes with specific function. For instance, Dobon et al. (2016) isolated RNA from infected wheat leaves at one to eleven days after inoculation (dai) with *Pst* and sequenced the samples on Illumina. They showed how the expression profile changes during infection. Sharma et al. (2018) also sequenced RNA that had been collected during various stages of the infection, this time with *Pt*. In addition, they compared plants with *Lr28* resistance gene and without it. They focused on gene expression in the host plant and elaborated on how one specific resistance gene affects the transcriptome profile. Similar study was also undertaken with *Pt* and wheat with and without *Lr57* (Yadav et al. 2016).

Next-generation sequencing enabled first identification of avirulence genes in rust pathogens, specifically *AvrSr35* and *AvrSr50* in *Pgt* (Chen et al. 2017; Salcedo et al. 2017). To identify *AvrSr35*, Salcedo et al. (2017) created mutants of *Pgt* race RKQQC, which is avirulent to wheat with resistance gene *Sr35*. To create the mutants, ethyl methane sulfonate (EMS) was used. Fifteen samples with gained virulence to *Sr35* were identified and their DNA was sequenced. 30429 mutations were found in the EMS mutants, but only one annotated gene carried mutations in all the 15 samples with the gained virulence to *Sr35*. This gene was then shown to code the avirulence factor that directly triggers the resistant response in plants by co-expressing it together with *Sr35* in *Nicotiana* leaves. Chen et al. (2017) identified *AvrSr50* by sequencing of *Pgt* accessions *Pgt*279 and *Pgt*632, where the former is avirulent to resistance gene *Sr50* and the latter is its spontaneous mutation that is virulent to the gene. They discovered a loss-of-heterozygosity event where the region that was missing in the virulent strain contained 24 predicted secreted protein genes. Those were the candidate genes that were tested by co-expression with the corresponding resistance gene *Sr50* in *Nicotiana benthamiana* leaves. The single gene that caused cell death response when co-

expressed with *Sr50* was designated *AvrSr50*. The two studies described above also showed that in nature, the *AvrSr35* and *AvrSr50* genes were disabled by insertions.

3. Aims of the thesis

Principal aim of this thesis was to analyze currently grown wheat cultivars registered in Czech Republic for stem rust resistance genes using molecular markers. The goal was to characterize the resistance, especially considering new pathogen races emerging from the tropical regions. The genotyping results obtained by using several types of markers were aimed to complement the results of field resistance trials and greenhouse trials undertaken with previously specified pathogen races.

Secondary aims were to

(i) further describe the resistance in a smaller subset of lines using adult plant disease phenotyping in controlled environment,

(ii) use machine learning to perform image analysis for phenotypic tests and establish this method for future phenotyping,

(iii) help explain rust resistance in previously tested spelt wheat cultivars, and

(iv) recommend existing lines and gene combinations to successfully combat virulence profiles of newly emerging pathogen races.

4. Hypotheses

Aims of the thesis were based on following hypotheses:

1) Novel pathogen races originating in tropics break down stem rust resistance in cultivars grown in Czech Republic.

2) There are unknown resistance genes present in local germplasm, some may be adult plant resistance genes. Therefore, there is a need for controlled adult plant resistance tests.

3) Recently reported markers linked to *Sr8a* and *Sr11* resistance genes can be used to detect those genes in local genetic backgrounds and those genes are still present and may be partly effective.

5. Materials and methods

5.1. Panels of winter wheat cultivars in marker assays

Genetic materials were sourced in form of seeds either from the gene bank of Crop Research Institute, Prague, or from the Central Institute for Supervising and Testing in Agriculture (ÚKZÚZ). Two panels of winter wheat cultivars grown in Czech Republic were assembled from the materials. First panel comprised of 58 cultivars that were selected in a way that they covered >80 % of the winter wheat seed propagation area reported in 2019 (Central Institute For Supervising and Testing In Agriculture 2019). This panel was used for STS, SCAR, and CAPS markers. Second, extended panel was used for KASP marker assays. This second panel comprised of 90 cultivars that were selected so that they covered at >80 % of the winter wheat seed propagation area reported in all years from 2014–2022. The idea behind covering most wheat growing area in the 9-year period was to determine temporal changes in gene presence frequencies in the agricultural landscape (Table 6).

Table 6: Panel of winter wheat cultivars with respective proportions in the winter wheat seed propagation area reported to the Central Institute for Supervising and Testing in Agriculture in years 2014–2022

Cultivar	2014	2015	2016	2017	2018	2019	2020	2021	2022
Adina	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.13%	0.33%
Advokat	0.00%	0.00%	0.00%	0.00%	0.06%	0.56%	0.53%	0.63%	0.53%
Akteur	1.29%	0.46%	0.11%	0.19%	0.07%	0.08%	0.00%	0.00%	0.00%
Annie	0.78%	1.68%	1.63%	1.68%	1.88%	1.65%	0.77%	0.31%	0.27%
Arkeos	2.02%	2.78%	2.20%	1.57%	1.71%	1.68%	2.15%	1.34%	1.17%
Arktis	1.50%	0.90%	0.74%	0.54%	0.28%	0.08%	0.00%	0.00%	0.00%
Askaban	0.00%	0.00%	0.00%	0.00%	0.00%	0.12%	0.21%	0.66%	1.05%
Asory	0.00%	0.00%	0.00%	0.00%	0.00%	0.12%	0.61%	1.11%	1.00%
Athlon	2.59%	1.77%	1.47%	1.37%	1.31%	0.84%	0.00%	0.00%	0.00%
Avenue	2.47%	2.51%	2.78%	3.05%	3.41%	3.05%	2.52%	2.28%	3.09%
Baletka	1.08%	0.88%	0.48%	0.30%	0.12%	0.00%	0.00%	0.00%	0.00%
Balitus	0.00%	0.08%	1.08%	1.02%	1.09%	0.96%	0.89%	0.73%	0.69%
Bardotka	0.70%	0.70%	0.75%	0.19%	0.17%	0.02%	0.02%	0.00%	0.00%
Bodyček	2.96%	1.65%	1.56%	1.13%	1.05%	0.85%	0.84%	0.84%	0.83%
Bohemia	7.11%	6.01%	4.14%	3.31%	2.46%	1.77%	1.23%	0.69%	0.44%
Bonanza	0.00%	0.09%	0.97%	1.32%	1.26%	1.45%	1.67%	1.85%	1.49%
Brilliant	0.68%	0.66%	0.75%	0.55%	0.50%	0.42%	0.45%	0.46%	0.31%
Butterfly	0.00%	0.00%	0.00%	0.04%	0.65%	2.06%	2.22%	1.46%	1.04%
Campesino	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.29%	0.53%
Chiron	0.00%	0.00%	0.00%	0.00%	0.07%	1.74%	1.14%	0.80%	0.48%
Cimrmanova raná	2.05%	0.89%	0.49%	0.19%	0.07%	0.00%	0.00%	0.00%	0.00%
Crossway	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.08%	0.32%
Dagmar	2.66%	2.65%	3.24%	3.36%	2.98%	2.71%	2.59%	2.53%	2.15%

Cultivar	2014	2015	2016	2017	2018	2019	2020	2021	2022
Diadem	0.93%	0.52%	0.06%	0.04%	0.08%	0.03%	0.00%	0.00%	0.00%
Elixer	0.17%	0.74%	1.03%	1.26%	1.50%	1.38%	0.96%	0.49%	0.47%
Elly	2.37%	2.05%	1.63%	0.83%	0.82%	0.65%	0.46%	0.26%	0.25%
Etana	0.96%	0.78%	0.33%	0.20%	0.14%	0.06%	0.00%	0.00%	0.00%
Evina	1.78%	2.14%	2.17%	1.93%	1.54%	0.58%	0.41%	0.36%	0.22%
Fakir	0.91%	1.17%	1.92%	2.08%	2.02%	2.06%	1.75%	1.44%	1.13%
Federer	1.48%	0.65%	0.32%	0.34%	0.03%	0.00%	0.00%	0.00%	0.00%
Fenomen	0.00%	0.00%	0.00%	0.07%	0.27%	0.94%	1.62%	2.69%	3.26%
Forhand	1.45%	0.57%	0.21%	0.01%	0.00%	0.03%	0.00%	0.00%	0.00%
Frisky	0.00%	1.45%	0.05%	1.58%	2.02%	2.84%	3.54%	3.23%	2.90%
Futurum	0.00%	0.00%	0.00%	0.09%	0.28%	0.53%	0.75%	0.49%	0.08%
Genius	4.28%	4.79%	6.06%	5.41%	4.78%	3.45%	2.85%	2.23%	1.84%
Golem	1.11%	1.59%	1.93%	1.02%	0.76%	0.41%	0.14%	0.21%	0.11%
Gordian	0.56%	1.34%	1.31%	1.48%	1.43%	0.72%	0.84%	0.46%	0.20%
Grizzly	0.11%	0.82%	0.59%	0.81%	0.69%	0.65%	0.73%	0.66%	0.37%
Illusion	0.00%	0.00%	0.00%	0.00%	0.00%	0.13%	1.02%	1.87%	2.31%
JB Asano	2.09%	1.51%	0.91%	0.30%	0.41%	0.30%	0.10%	0.08%	0.11%
Johnson	0.00%	0.00%	0.00%	0.00%	0.00%	0.40%	2.43%	3.00%	3.68%
Judita	0.00%	0.00%	1.13%	1.78%	0.81%	0.18%	0.00%	0.02%	0.00%
Julie	1.51%	4.49%	5.44%	5.78%	6.89%	5.68%	5.18%	5.32%	5.12%
Kalbex	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	1.28%
KWS Donovan	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.13%	0.30%
KWS Elementary	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.32%	1.22%	1.02%
Lear	0.70%	1.00%	1.05%	0.95%	0.80%	0.73%	0.39%	0.37%	0.28%
LG Absalon	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.15%	0.96%	2.50%
LG Dita	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.14%	0.54%	0.91%
LG Imposanto	0.00%	0.00%	0.00%	0.37%	1.57%	2.42%	1.75%	0.83%	0.55%
LG Keramik	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.87%	1.23%
LG Mocca	0.00%	0.00%	0.00%	0.00%	0.00%	1.69%	3.76%	5.80%	5.19%
LG Mondial	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.80%
LG Orlice	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.54%	1.15%	1.01%
Magister	1.53%	0.57%	0.12%	0.12%	0.00%	0.00%	0.00%	0.00%	0.00%
Matchball	2.01%	2.35%	2.55%	1.53%	0.95%	0.70%	0.21%	0.13%	0.18%
Megan	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.07%	1.28%	2.12%
Mercedes	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.15%	0.35%
Mulan	1.62%	1.05%	0.75%	0.42%	0.13%	0.00%	0.00%	0.00%	0.00%
Nelson	1.07%	1.04%	1.53%	0.35%	0.53%	0.04%	0.04%	0.00%	0.00%
Nordika	1.21%	2.06%	0.76%	0.45%	0.14%	0.08%	0.00%	0.00%	0.00%
Ostroga	0.91%	0.66%	0.80%	0.36%	0.00%	0.00%	0.00%	0.00%	0.00%
Pannonia NS	2.43%	2.09%	1.68%	1.38%	1.26%	0.00%	0.00%	0.00%	0.00%
Papageno	1.72%	0.79%	0.28%	0.22%	0.13%	0.10%	0.06%	0.00%	0.00%
Partner	0.00%	0.00%	0.00%	0.00%	0.11%	0.30%	0.24%	0.22%	0.20%

Cultivar	2014	2015	2016	2017	2018	2019	2020	2021	2022
Patras	2.73%	3.23%	3.03%	2.93%	2.93%	2.78%	1.96%	1.37%	0.70%
Penelope	0.00%	0.00%	0.54%	0.91%	0.68%	0.32%	0.05%	0.02%	0.05%
Pirueta	0.00%	0.00%	0.00%	0.00%	0.00%	0.61%	1.45%	1.45%	1.04%
Ponticus	0.00%	0.00%	0.00%	0.53%	2.42%	3.84%	4.01%	3.76%	3.69%
Potenzial	2.51%	1.90%	0.50%	0.13%	0.03%	0.05%	0.03%	0.00%	0.04%
Proteus	0.00%	0.00%	0.00%	0.07%	0.26%	0.34%	0.26%	0.11%	0.17%
Rebell	0.06%	1.51%	3.47%	2.82%	1.94%	1.19%	0.90%	0.54%	0.43%
RGT Premiant	0.00%	0.00%	0.00%	0.45%	1.32%	0.38%	0.00%	0.00%	0.00%
RGT Reform	0.00%	0.16%	3.86%	7.52%	7.03%	6.87%	5.69%	4.92%	4.59%
RGT Sacramento	0.00%	0.00%	0.11%	0.76%	1.15%	1.40%	2.16%	2.20%	1.93%
Rivero	0.00%	0.00%	1.07%	3.50%	3.35%	2.59%	1.33%	0.57%	0.33%
Sailor	0.90%	0.61%	0.33%	0.27%	0.05%	0.00%	0.00%	0.00%	0.00%
Seladon	1.26%	0.79%	0.40%	0.13%	0.10%	0.00%	0.00%	0.00%	0.00%
Sheriff	0.00%	0.00%	0.02%	0.24%	0.65%	1.12%	0.93%	0.83%	0.51%
Skif	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.23%	0.75%
Sofru	0.05%	0.04%	0.18%	0.35%	0.54%	1.22%	1.44%	1.62%	1.54%
Steffi	0.00%	0.00%	0.00%	0.69%	1.17%	1.31%	1.34%	0.77%	0.31%
SU Tarroca	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.89%
Tiguan	1.02%	0.42%	0.24%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%
Tobak	5.46%	6.50%	8.07%	5.79%	3.41%	2.48%	1.38%	0.91%	0.43%
Turandot	3.07%	2.39%	2.59%	2.35%	2.50%	2.25%	1.93%	1.94%	1.60%
Vanessa	2.63%	3.37%	3.50%	2.97%	2.98%	2.60%	2.17%	1.68%	1.15%
Viki	0.00%	0.00%	0.11%	0.80%	1.29%	1.08%	0.36%	0.31%	0.13%
Viriato	0.05%	0.46%	1.33%	2.37%	3.33%	4.64%	5.39%	4.48%	4.22%
WPB Calgary	0.00%	0.00%	0.00%	0.00%	0.07%	0.26%	0.29%	0.15%	0.33%
total area	**80.54%**	**81.31%**	**86.35%**	**86.62%**	**86.43%**	**84.59%**	**81.41%**	**80.58%**	**80.52%**

5.2. Spelt wheat panel

Additionally, a panel of 16 spelt wheat cultivars (*Triticum spelta*) was also selected for marker assays. The cultivars are a subset of a larger panel of 80 winter spelt genotypes from the FP7 project HealthyMinorCereals used for disease trials in Czech Republic and other European countries in years 2013–2017. The diversity panel included historic and current European winter spelt germplasm. The 16 cultivars were chosen based on their lower susceptibility to rusts in those trials with the aim of explaining their lower susceptibility by possible presence of known resistance genes used in winter wheat breeding. Since the study was also covering other rusts than stem rust, marker assays for *Lr1, Lr9,* and *Lr10* are also included, however further information on those is not presented here and can be found in the corresponding article (Dumalasová et al., currently under review).

5.3. Reference lines

Reference wheat cultivars previously reported to carry the resistance alleles were used as positive controls. For genes used in the American differential set, the corresponding lines were also used. Eagle, Hope, Redman, and Pembina were used for *Sr2*. McMurachy, Gatcher, Shield, and ISr6-Ra were used as positive controls for *Sr6* marker (McIntosh et al. 1995). For *Sr8a*, Asta, Boka, Hereward, and Tremie were picked based on postulation by Pathan and Park (2007). Gabo, Timstein, and Trident were used as positive controls for *Sr11* (Nirmala et al. 2016). Agra was used as a reference for *Sr36*. Brigadier was used as a reference for the wheat-rye translocation that contains *Sr31* (Rabinovich 1998).

5.4. Marker assays

Seeds were sown in pots and grown until seedling stage. Total plant DNA was isolated from the first two developed leaves using a DNeasy Plant Mini Kit according to the manufacturer's instructions (Qiagen GmbH, Germany). The set of markers used in this study was selected based on the availability of the marker, importance of the gene or knowledge of its previous usage in breeding programs, and availability of lines to be used as positive controls. Markers were used in PCR assays with reaction mixtures and cycles according to the original sources of the individual markers (Table 7). Reaction mixtures included Qiagen Taq polymerase and PCR buffer. Oligonucleotides used as primers were supplied by Generi Biotech, Czech Republic. In case of the CAPS marker csSr2, the PCR product was digested by a restriction enzyme FastDigest BspHI according to manufacturer's instructions (Fermentas, Lithuania). All PCR products were visualized on agarose gels stained with ethidium bromide using a GeneRuler 100 bp DNA Ladder as a reference for nucleotide lengths (Thermo Scientific, USA), and scored according to the corresponding sources in literature.

Table 7: Molecular markers used in PCR assays. Reaction mixes and conditions are available in respective sources.

Gene	Marker	Type	Primer sequences	Amplicon size	Source
Sr2	**csSr2**	CAPS	5'-CAAGGGTTGCTAGGATTGGAAAAC-3'	172, 112, 53 bp*	Mago et al. (2011)
			5'-AGATAACTCTTATGATCTTACATTTTTCTG-3'		
Sr6	**Xcfd43**	SSR	5'-AACAAAAGTCGGTGCAGTCC-3'	215 bp	Tsilo et al. (2009)
			5'-CCAAAAACATGGTTAAAGGGG-3'		
Sr15	**STS638**	STS	5'-CACAGCGATGAAGCAATGAAA-3'	542 bp	Neu et al. (2011)
			5'-GTCCAGTTGGTTGATGGAAT-3'		
Sr22	**XcsIH81-BM**	STS	5'-TTCCATAAGTTCCTACAGTAC-3'	257 bp	Periyannan et al. (2011)
			5'-TAGACAAACAAGATTTAGCAC-3'		
	XcsIH81-AG	STS	5'-CTACCTCTGTCAATTTGAAC-3'	**	Periyannan et al. (2011)
			5'-GAAAAATGACTGTGATCGC-3'		
Sr24	**SCS1326**	SCAR	5'-GCATCGTGCAGCTAGTTCTG-3'	613 bp	Gupta et al. (2006a)
			5'-AGGCATCGTGAAAAGAGAACA-3'		
Sr25	**SCS265**	SCAR	5'-GCGGATAAGCAGAGCAGAG-3'	512 bp	Gupta et al. (2006b)
			5'-GGCGGATAAGTGGGTTATGG-3'		
Sr31	**iag95**	STS	5'-CTCTGTGGATAGTTACTTGATCGA-3'	1,100 bp	Mago et al. (2002)
			5'-CCTAGAACATGCATGGCTGTTACA-3'		
Sr36	**Xwmc477**	SSR	5'-CGTCGAAAACCGTACACTCTCC-3'	187 bp	Tsilo et al. (2008)
			5'-GCGAAACAGAATAGCCCTGATG-3'		
Sr38	**VENTRIUP**	STS	5'-AGGGGCTACTGACCAAGGCT-3'	259 bp	Helguera et al. (2003)
	LN2		5'-TGCAGCTACAGCAGTATGTACACAAAA-3'		

***after digestion of the initial product with a *BspHI* restriction enzyme. **second primer pair amplifies negative allele**

KASP markers for prediction of *Sr8a*, *Sr11*, *Sr15*, and *Sr36* were selected for assays with the extended set of 90 cultivars (Table 8). These markers were selected because the presence of the linked genes can be expected in European wheat as they have already been reported in numerous commercial cultivars, not only in research or unreleased lines. The markers wsnp_Ku_c39334_47795461 and wsnp_Ra_c3996_7334169 were mapped within 1.83 cM of *Sr8a* in a biparental population of spring wheat lines (Guerrero-Chavez et al. 2015). These markers remain to be tested in a panel of commercial wheats. The first of those markers was also used in another mapping study (Hiebert et al. 2017) where it co-segregated with xcalibur_c431_1130, located 2.2 cM from what was presumably *Sr8a*. Additionally, Hiebert et al. (2017) report linkage between kwh54 (also Excalibur_c12085_276, IWB22036) and the resistance in cultivar Harvest which is probably *Sr8a*. Therefore, this marker was also selected for my study. Markers KASP_6BL_IWB1072 and KASP_6BL_IWB7247 were selected as they were reported to be linked to *Sr11* (Nirmala et al. 2016). These have already been tested on panels of wheat lines from three

different continents, and they accurately predicted the presence of the resistance gene. The first is based on a SNP BS00074288_51, the second is based on Tdurum_contig55744_822. The marker Sr36/Pm6_8068 selected for *Sr36* is based on CIMwMAS0167 SNP and has already been used in various breeding programs (i.e., https://excellenceinbreeding.org/toolbox). *Sr15* marker IWB30995 was selected based on its reported linkage in mapping studies (Babiker et al. 2015; Gao et al. 2019).

For the KASP marker assays, markers (Table 8) were supplied as KASP-by-design (KBD) primer mixes (LGC Genomics, Berlin, Germany) based on corresponding SNP sequences. Reaction mixtures were prepared with high-ROX master mix and thermal cycling conditions were set up according to guidelines provided by the manufacturer. Reactions mixes of 10 µl final reaction volume contained 5 µl of KASP master mix, 0.14 µl of primer mix, 2 µl of DNA sample, and sterile water. Allele quantification and discrimination was performed on ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, United States) in suitable MicroAmp™ Optical 96-well reaction plates manufactured by the same company. Plates were enclosed with optically clear seals. Alternatively, a Bio-Rad CFX Connect (Bio-Rad Laboratories Inc., Hercules, CA, United States) was used.

Fluorescence of FAM and HEX fluorophores was measured to detect presence of allele X and / or allele Y in the DNA samples, and samples were automatically divided in clusters on allele discrimination plots. If the reactions were performed on ABI 7900HT, ROX fluorescence was also measured for the calibration of reads. All calls were then checked manually. If samples were difficult to assign to clusters, either further cycles were added, or the protocol was repeated with altered thermal cycling conditions. Troubleshooting guide provided by LGC Genomics was used when necessary.

Table 8: SNP sequences for KASP assays

Marker name	Gene	AlleleX	AlleleY	SNP sequence in [allele1/allele2] format
IWA7913 (wsnp_Ra_c3996_7334169)	***Sr8a***	A	G	AGCAGCTACGTTTGACATCAGATTGCCAAACATCGGGTTGTTGAGAACACTTGATAGCATACTGCCGAGGTCAACTTGCCCTTCACCACCCGGTCTTCTC**[A/G]**GAGGCATTCCAGCAGCAGACCGAGGAGTTGAAGATGGAGCTTGAGAAGCCGTGACACTGCCGCTGCTCGCATTAGCACCTCTTGAAACAAGAGCTTGCAG
wsnp_Ku_c39334_47795461	***Sr8a***	A	G	AGCTGTTGATTTATCAACTTTTTACACCGAAATCCCCTGCTTGTCAGCAAAAGYAGAGGTTGTTATTCATGGTGAATTTACACATGTTACACACAAAACC**[G/A]**CAGACATCATTGGATTTGGAGGGGCCACTTGCTCATCATCTTTACTGTGGATGCCGACTGTGGCTGGGTGCCGTTCCAGCAAGCCAAAAACTGTTAGAGA
IWB22036 (Excalibur_c12085_276)	***Sr8a***	A	G	TTCATGAGTGCGCTACTTGGGGCACTATTCGCTCAGTATCTGTTCTTCTT**[A/G]**GGCTTGAGCTACACCACTGCAACACTGGCTGCAACTGTCTCCAATATGAC

Marker name	Gene	AlleleX	AlleleY	SNP sequence in [allele1/allele2] format
6BL_IWB72471 (Tdurum_contig55744_822)	***Sr11***	C	A	TCTCAACTGATCTAAGTTCCTTTGTCAATTCATTGTGGGACAACTTCACC[**A/C**]CATCTAGTTTATCCTTTAGCTTTGAGTTTTCAGTGCGAACTTCTGCCAGT
6BL_IWB10724 (BS00074288)	***Sr11***	A	G	GGAGGAAACAGACTATTGGAAAACCGTCATCTCGCGTATGTAGGGATGCT[**A/G**]TTTCAGCTAAGGTATCTCAACATTTACATGACAGCAGTAAGCGAGCTCCC
Sr36/Pm6_8068	***Sr36***	T	C	GGATGAGCCACCCAAGCTGTTCCACTTCTGGGGYCGCATCAAAATCTGTACCCTCAAGACAAAAGAAGTCGACAAAGGGCAGTGCTTATGACGACGATGA[**T/C**]GATGCGTATAATGATGAGAATGACAATGACGACGATGAYGACGATGACGCAGATGAAGATGATCCAGATGATGTGGATTTTGAGCCAGACAGTGAAACCG
IWB30995	***Sr15/Lr20/Pm1***	A	G	ACATAAGGCGCAGTTGCAACAGCTCTGAATCTACACCTACATGGGAGAAC[**A/G**]CTACTAAGTAGCCTATAGCACGGCCACCCGATCTCTGGACGAACAGTCGG

5.5. Field disease trials

Field resistance tests were carried out in the locality Prague – Ruzyně (50.087441 N, 14.298697 E, 376 m above sea level) in an artificially infected field, with a mixture of stem rust isolates that were collected in previous years. Each cultivar was grown in three-row microplots 0.5 m long and 0.6 m wide in two replicates. Seeds of the cultivars were provided by ÚKZÚZ or gene bank of the CRI. The inoculum for the experiments was prepared in the greenhouse conditions from urediospores on leaves collected during previous growing seasons to simulate a range of races that appear naturally. The inoculum was a water suspension containing a mixture of unspecified races that previously occurred in the location. When plants reached growth phase BBCH 33 – 36, the water suspension of urediospores was injected into the stems of the susceptible variety Michigan Amber (spreader), from where the infection spread to the test materials. The density of spores in water suspension was 100 – 120 mg/l. Symptoms were evaluated on three dates on a scale of 1 to 9 (1 - resistant; 9 - susceptible). These field trials are undertaken at Crop Research Institute on a yearly basis and author of this thesis took part during the years of his doctoral studies.

5.6. Subset of lines for seedling and adult plant resistance phenotyping

Six cultivars of particular interest were picked for a more detailed phenotypic study of their response to stem rust. These six lines were selected based on previous genotypic and phenotypic results with an intention to further explain their resistance patterns. Additionally, Michigan Amber was used as a universally susceptible control in the experiments (Table 9).

Table 9: Cultivars used for adult plant phenotyping.

	Cultivar	Postulated genes	Registered in Czech Republic	Owner
1	**Arkeos**	none found	Not in register	Limagrain Central Europe Cereals, s. r. o.
2	**Campesino**	*Sr24, Sr38*	2021	SECOBRA Saatzucht GmbH
3	**Evina**	*Sr38*	2012	Limagrain Central Europe Cereals, s. r. o.
4	**LG Mocca**	none found	2019	Limagrain Central Europe Cereals, s. r. o.
5	**Rivero**	*Sr38*	2016	NORDSAAT Saatzucht GmbH, DE
6	**RGT Reform**	*Sr38*	Not in register	Société RAGT 2n, FR
7	**Michigan Amber**	susceptible control	Not in register	susceptible control

LG Mocca was selected as the most widely grown cultivar in the year 2022 according to its share of the winter wheat propagation areas. Furthermore, this line was previously found to be highly susceptible to stem rust in field conditions (unpublished data). Cultivar Campesino was selected due to its high resistance and postulated combination of *Sr24* and *Sr38* genes. Arkeos was also selected as a highly resistant cultivar, however the resistance genes in this case are unknown. None of the prevalent genes were detected in this cultivar. Cultivars Evina, RGT Reform, and Rivero were selected because of their variable reaction to distinct pathogen races. The experiments were undertaken to explain such discrepancies into more detail and describe the adult plant resistance in a controlled environment.

5.7. *P. graminis* isolates for phenotypic tests

Two *Pgt* isolates were selected for greenhouse experiments. These two isolates have distinct virulence patterns that were analysed using the North American differential set (Table 10). Their designations are TKRPF and PHRTF. These two races were used to inoculate the subset of 58 wheat cultivars previously tested with molecular markers to validate the presence of some of the tested genes. They also represent typical virulence patterns of *Pgt* races that appeared frequently in Czech Republic during writing of this work. They are avirulent to *Sr24* and *Sr31*, which is typical for the local pathogen population, however, they show virulence to *Sr38*, which aligns with the current virulence shift in Europe. Notably, both are avirulent to *Sr11*, and TKRPF has additional virulence on *Sr21* and *Sr8a*.

Table 10: Virulence patterns of the Puccinia graminis isolates used for phenotypic experiments. H: high virulence, L: low virulence to each of the Sr genes in the differential set.

Race code	*5*	*21*	*9e*	*7b*	*11*	*6*	*8a*	*9g*	*36*	*9b*	*30*	*17*	*9a*	*9d*	*10*	*Tmp*	*24*	*31*	*38*	*McN*
TKRPF	H	H	H	H	L	H	H	H	H	H	L	H	H	L	H	H	L	L	H	H
PHRTF	H	L	H	H	L	H	L	H	H	H	L	H	H	H	H	H	L	L	H	H
RCHPC	H	H	L	H	L	L	L	H	L	H	L	H	H	L	H	H	L	L	L	H
TRTTF	H	H	H	H	H	H	L	H	H	H	H	H	H	H	H	H	L	L	H	H

Another two pathogen isolates of *Pgt* collected in 2019 growing season were selected for further phenotypic experiments. Their race designations were determined on a differential set of near-isogenic lines (testing lines with single resistance gene each) as RCHPC (Figure 5) and TRTTF (Figure 6). RCHPC has a virulence combination that resembles older isolates from central Europe, while TRTTF is a virulence pattern that is often seen in incursions from tropics. The isolates were, however, not genotyped, so assignment to a genetic group cannot be made. The idea behind picking these two races was their contrasting virulence profile, with the TRTTF race probably belonging to tropical incursions that are currently starting to dominate the European region. The most significant difference between the isolates is the virulence on *Sr6*, *Sr11*, *Sr36*, and *Sr38* in TRTTF. Both isolates are avirulent to *Sr8a*, *Sr24*, and *Sr31*. Isolates were multiplied on susceptible cultivar Michigan Amber in the greenhouse to provide enough urediospores for inoculating both seedling and adult plants in the experiments.

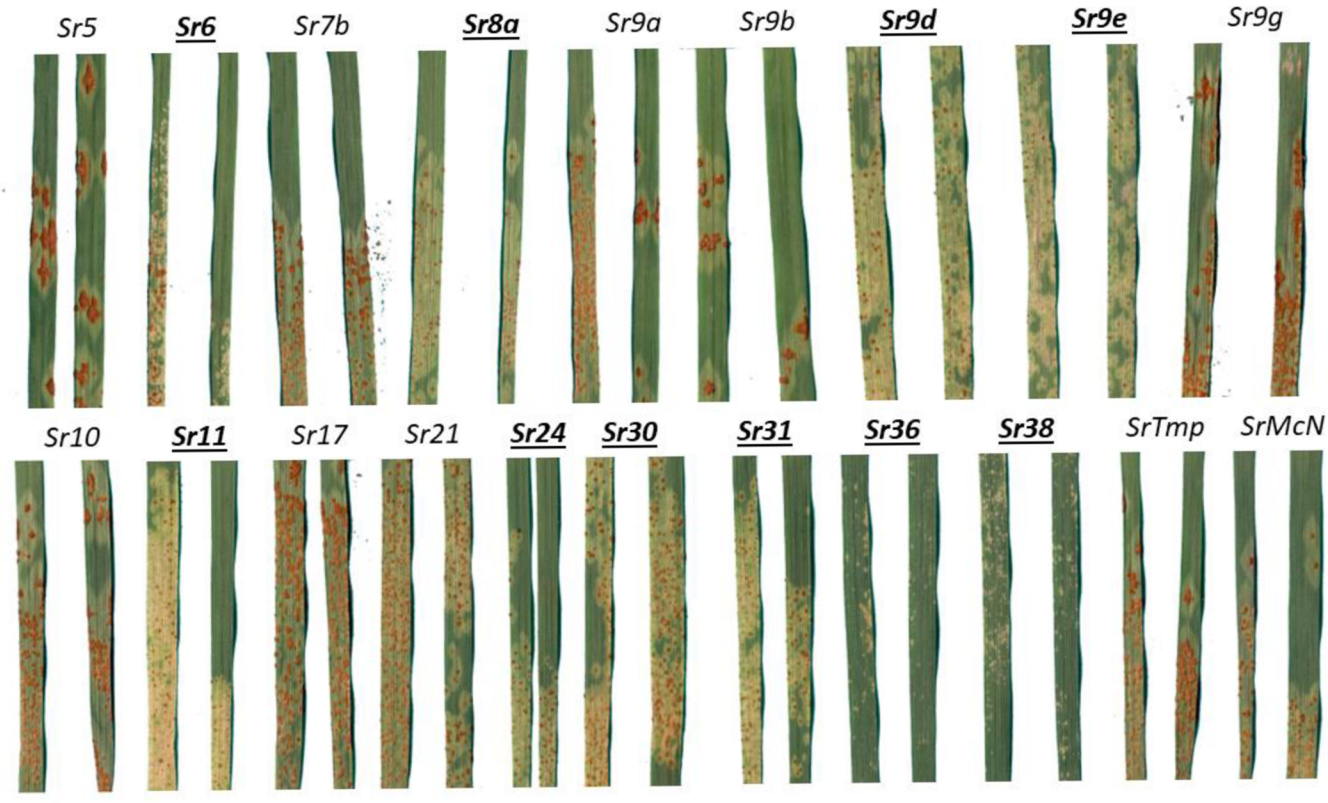

Figure 5: Disease symptoms of Puccinia graminis f. sp. tritici race RCHPC on a differential set of wheat near isogenic lines (test lines with a single resistance gene). Each isogenic line is represented by two seedling leaf segments. Avirulent (incompatible) reactions are labelled in bold and underlined.

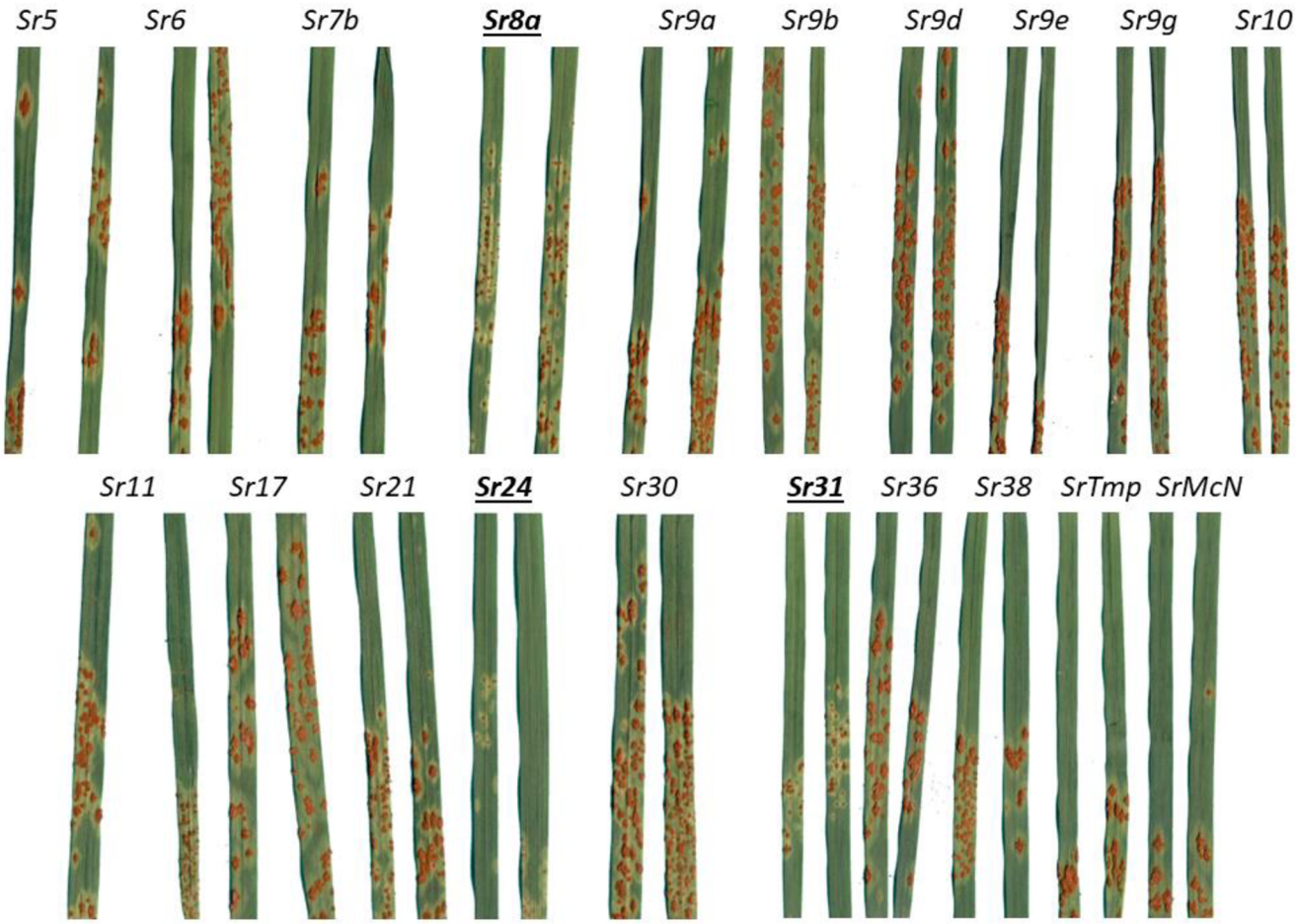

Figure 6:Disease symptoms of Puccinia graminis f. sp. tritici race TRTTF on a differential set of wheat near isogenic lines (test lines with a single resistance gene). Each isogenic line is represented by two seedling leaf segments. Avirulent (incompatible) reactions are labelled in bold and underlined.

For adult plant phenotyping, seeds of the winter wheats were first germinated in a petri dish filled with wet filter paper in 4°C. The seeds that successfully germinated were planted in pots in 5 repetitions. Total number of plants per one treatment (i.e., inoculation with one isolate) was then 35. The plant pots were randomly distributed in a plastic container and carefully labelled. At this point the containers were transferred to a cold chamber under 4°C for 6 weeks for thorough vernalization. After vernalization, the containers were transferred to a cultivation chamber with 24°C/17°C for 12/12 h, with 22 hours of artificial light / two hours of darkness. Plants were watered regularly, and any emerging tillers were trimmed so that only the main stem remained. Plants were grown until reaching adult plant stage, approximately 40 – 65 on the Zadoks scale (Zadoks et al. 1974) and the growth stage was recorded for each plant individually before inoculation.

Containers with plants were then enclosed in a 150x120x70 cm frame covered with a transparent plastic film to prevent contamination between the treatments. This enclosing allowed for water condensation inside the frame after increasing the air humidity for the inoculation, while also

allowing light in. A suspension of urediospores in distilled water containing a drop of Tween 20 was prepared at a concentration of roughly 250 000 spores / 1 ml. Suspension was applied to plants using an atomizer, after which the light was turned off for 16 hours to allow germination of spores. The plants were then kept in 16 hours of light and 8 hours of darkness every day for the disease development. The top part of the plastic film was removed from the frame 24 hours after inoculation since the maximum air humidity is no longer necessary when the mycelium is developing inside the plant tissues.

Disease symptoms were observed 12 days after inoculation on a 0–9 scale where 0 means no disease symptoms and 9 means maximum severity. Flag leaves and flag-1 leaves were scored separately. Both flag leaves and flag-1 leaves were then collected and used for further analysis.

5.8. Image analysis by machine learning

Five replicates of 10 cm long segments of leaves for each treatment were fixed onto a sheet of paper using a double-sided tape so that the abaxial side was facing up. Sheets were scanned in 600 dpi resolution using an image scanner. All images were scanned with identical settings and were never processed individually. Trainable Weka Segmentation plug-in v.3.3.2 (Arganda-Carreras et al. 2017) of Fiji image processing distribution of ImageJ (Schindelin et al. 2012) was used to segment the images into classes. The classifier was first trained on a training collage of representative phenotypes, including both seedling and adult leaf segments and a full range of disease symptoms. It was trained to recognize four classes: (i) uredia, (ii) chlorosis, (iii) healthy leaf area, and (iv) background. The result of processing the training image was then observed and additional examples of the four classes were added as needed to achieve a very precise distinction between the classes (Figure 7). Afterwards the classifier was saved and applied to the full set of images. For each treatment, the quantities of pixels separated into the four classes were extracted using the list view of the histogram function. After this point only relative quantities of the disease symptoms were evaluated: (i) portion of leaf area covered by pustules, (ii) ratio of leaf area covered by chlorosis, (iii) ratio of uredia area to chlorosis area.

Leaves that were scanned were subsequently used for quantitative analysis.

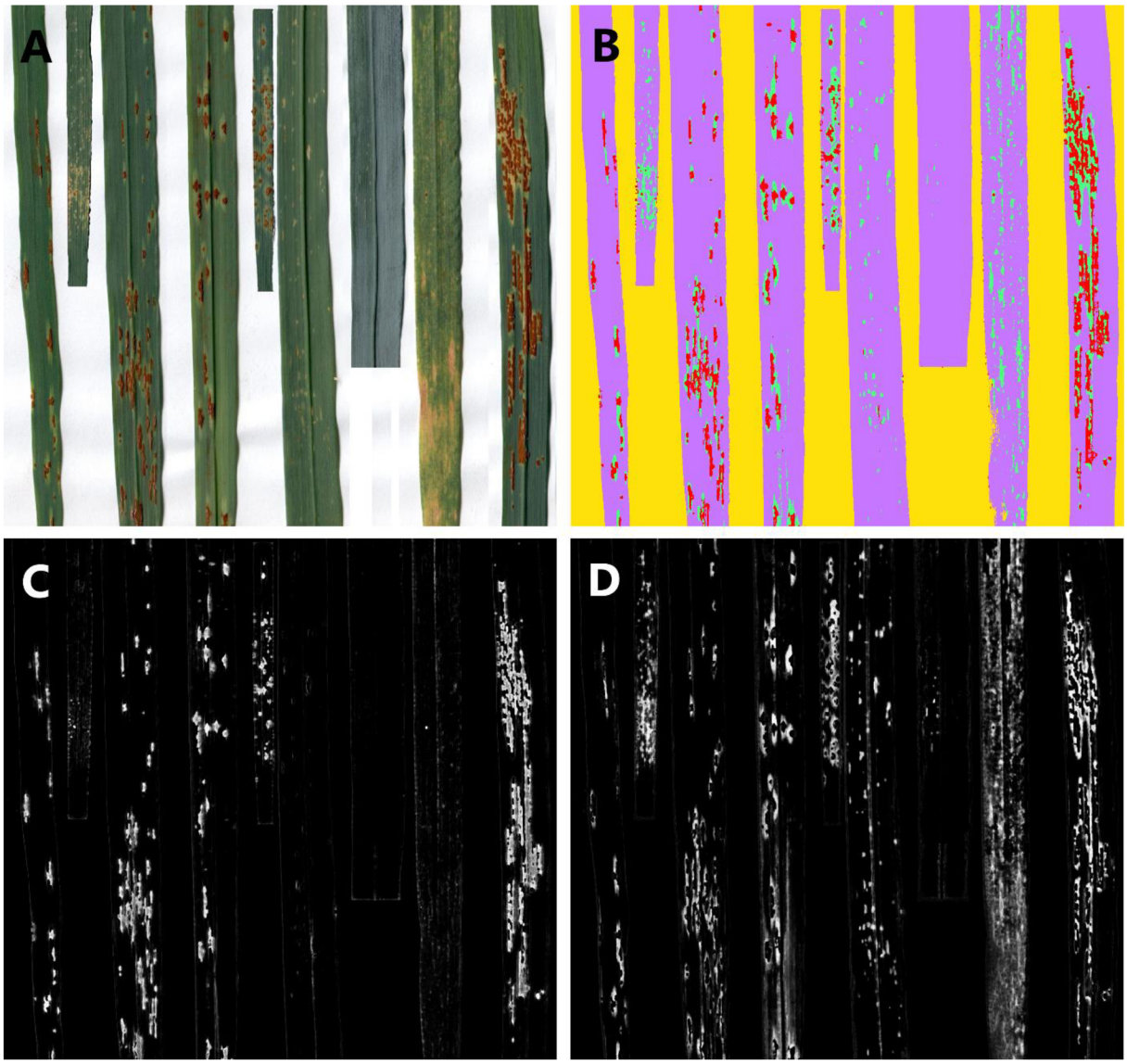

Figure 7: Process of training a classifier in Trainable Weka Segmentation plug-in of ImageJ. (A) Collage of scanned leaf images with various disease phenotypes used for training the classifier; (B) Training image classified with the trained model where pustules are in red, chlorosis in green, healthy leaf in purple, and background in yellow; (C) probability map of a pustule class (lighter pixels indicate higher confidence in classifying the area as a pustule); (D) probability map of a chlorosis class

5.9. Fungal tissue quantification

Amounts of fungal tissue in the previously scored plant parts were compared between treatments following a WAC assay method (Ayliffe et al. 2013) with slight modifications. If there were several repetitions of a sample or several leaves in a single sample, those were bulked. Then the infected plant tissues were weighted, ground into a very smooth powder in liquid nitrogen using a pestle and mortar and transferred to 2 ml Eppendorf tubes or Falcon tubes. Samples were suspended in 1M KOH and cultivated in 40°C for 18 hours. The hydroxide was then rejected, and samples were neutralized in 0.05M Tris-HCl (pH = 7.0) at a rate of 5 ml per 1 mg of weighted sample. 100 µl of each sample was transferred to a 200 µl tube in three replicates. 5 µl of wheat germ agglutinin

conjugated to fluorescein isothiocyanate (WGA-FITC, 1 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) was added to each sample and samples were incubated in RT for 1 h. Samples were then washed three times with Tris-HCl and transferred to microplates suitable for fluorometry. Fluorescence was measured using a Biotek Cytation 3 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The settings were 485 nm for excitation and 530 nm for emission, the reads were repeated three times.

5.10. Statistical analysis

Measured data from WAC assays were analysed separately for seedlings and adult plants, since the samples were processed separately and measured on separate microplates. Therefore, two linear mixed models were fitted using the lme4 package 1.1-31 (Bates et al. 2015) in R 4.1.2 (R Core Team 2021). In the models, wheat cultivar and genotype of the pathogen were considered as fixed effects with an interaction. In case of adult plants, flag leaves and flag - 1 leaves were measured separately, so the flag variable was also considered as a fixed effect. Because the reads were repeated three times for the same plate, a nested structure was introduced for the random effects with the individual reads nested inside technical replications. The sizes of fixed effects were compared post-hoc using Tukey's honestly significant differences (HSD) in R package multcomp 1.4-20 (Hothorn et al. 2008).

All graphs were plotted using ggplot2 package in R (Wickham 2009).

6. Results

6.1. Molecular marker assays and phenotyping

DNA samples of 58 varieties were tested for presence of 8 genes using STS, SCAR, and CAPS markers. All those markers showed clear and replicable results except marker for *Sr22* which only amplified a product indicating a negative result, including five additional varieties with previously reported *Sr22* allele presence: 'Aroona', 'Marquis', 'Steinwedel', 'Waldron', and 'Warigal'. Therefore, no results are included for *Sr22*. Markers for *Sr2*, *Sr25*, and *Sr36* were successfully optimized but showed only negative results in our selection, suggesting that these resistance genes are present in none of the 58 cultivars. Additionally, *Sr36* marker *Xwmc477* amplified both the negative and positive allele in our positive control 'Agra' (Figure 8), suggesting heterozygosity or contamination in the DNA sample. The markers showing only negative results are not included in Table 11 for redundancy.

Genes *Sr6*, *Sr24*, *Sr31*, and *Sr38* were detected with various frequencies (Table 11). Marker for *Sr6* was only present in one cultivar – 'Viriato' (Figure 8), *Sr24* was detected with a 13.79% frequency, *Sr31* with a 10.34% frequency. The positive allele for *Sr38* was amplified most frequently, in 63.79% of the samples. Six cultivars were tested positive for two resistance genes at once. None of the samples were positive for more than two resistance genes at once. According to these results it was possible to divide the cultivars into seven groups according to their resistance gene composition. All stem rust resistance field tests for all cultivars in the study from years 2014–2020 were grouped by major gene composition and their variance was analysed by ANOVA. Groups showed statistically significant differences ($F = 32.8$; $p < 0.001$). For cultivars with no Sr genes detected in this study, the mean of resistance trial results was 7.118. For cultivars with 1 Sr gene detected the mean was 3.309, for cultivars with 2 Sr genes the mean was 2.094.

Table 11: Results of four molecular marker assays on a set of 58 wheat cultivars. Column "Genes" lists genes that are assumed to be present in the cultivars based on the marker assays. Table includes results of greenhouse phenotypic tests with two Puccinia graminis races scored according to Stakman et al. (1962).

Cultivar	Molecular marker				Genes	Greenhouse tests*	
	Xcfd43	SCS1326	iag95	Ventriup / LN2		TKRPF**	PHRTF***
Advokat	- (185bp)	- (null)	+ (1100bp)	- (null)	*Sr31*	;1	;
Angelus	- (185bp)	- (null)	- (null)	- (null)	none	;2	3
Annie	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	3-	4
Apostel	- (185bp)	- (null)	- (null)	- (null)	none	3-	3
Arkeos	- (185bp)	- (null)	- (null)	- (null)	none	0	0
Askaban	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	3	4
Asory	- (185bp)	+ (607bp)	- (two amplicons)	+ (259bp)	*Sr24*, *Sr38*	0	;1

Cultivar	Molecular marker				Genes	Greenhouse tests*	
	Xcfd43	SCS1326	iag95	Ventriup / LN2		TKRPF**	PHRTF***
Athlon	- (185bp)	+ (607bp)	- (null)	- (null)	*Sr24*	;1-2	0;
Avenue	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	3	4
Balitus	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	3	3
Bernstein	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	3	4
Bodyček	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	3	3
Bohemia	- (185bp)	- (null)	- (null)	- (null)	none	;2	3
Bonanza	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	2-3	3-
Brilliant	- (185bp)	- (null)	+ (1100bp)	- (null)	*Sr31*	;1	0;
Butterfly	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	3	3
Campesino	- (185bp)	+ (607bp)	- (two amplicons)	+ (259bp)	*Sr24*, *Sr38*	;	0
Chiron	- (185bp)	- (null)	- (null)	- (null)	none	0	0;
Dagmar	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	3	3
Elixer	- (185bp)	+ (607bp)	- (null)	- (null)	*Sr24*	;1	;2
Elly	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	3	3
Evina	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	;	3
Fakir	- (185bp)	- (null)	- (null)	- (null)	*Sr38*	4	3
Fenomen	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	2-3	4
Frisky	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	3	3
Futurum	- (185bp)	+ (607bp)	- (null)	- (null)	*Sr24*	;	;
Gaudio	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	3	3-
Genius	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	3	3
Golem	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	3	3
Gordian	- (185bp)	+ (607bp)	- (null)	- (null)	*Sr24*	3-	;2
Grizzly	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	3	3
Hyfi	- (185bp)	+ (607bp)	- (null)	- (null)	*Sr24*	3	;
Illusion	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	3-	3
Johnson	- (185bp)	- (null)	+ (1100bp)	- (null)	*Sr31*	;	;
Julie	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	;2	3
Lear	- (185bp)	- (null)	- (null)	- (null)	none	3	4
LG Imposanto	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	3	4
LG Mocca	- (185bp)	- (null)	- (null)	- (null)	none	3	4
Matchball	- (185bp)	- (null)	+ (1100bp)	+ (259bp)	*Sr31*, *Sr38*	0	;1
Norin	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	4	4
Patras	- (185bp)	- (null)	- (null)	- (null)	none	2-3	3
Penelope	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	;	3
Pirueta	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	3	4
Ponticus	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	;2	3
Rebell	- (185bp)	- (null)	+ (1100bp)	+ (259bp)	*Sr31*, *Sr38*	0	0
RGT Premiant	- (185bp)	- (null)	- (null)	- (null)	none	2-3	4

Cultivar	Molecular marker				Genes	Greenhouse tests*	
	Xcfd43	SCS1326	iag95	Ventriup / LN2		TKRPF**	PHRTF***
RGT Reform	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	3	0;
RGT Sacramento	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	4	3
Rivero	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	;1-2	3
Sally	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	3	3
Sheriff	- (185bp)	+ (607bp)	- (null)	- (null)	*Sr24*	;1	;2
Sofru	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	3-	4
Steffi	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	;2	3
Tobak	- (185bp)	- (null)	- (null)	- (null)	none	3-	3
Turandot	- (185bp)	- (null)	- (null)	- (null)	none	;2	3-
Vanessa	- (185bp)	- (null)	+ (1100bp)	+ (259bp)	*Sr31*, *Sr38*	;	;1
Viki	- (185bp)	- (null)	- (null)	+ (259bp)	*Sr38*	;2	4
Viriato	+ (215bp)	- (null)	- (null)	+ (259bp)	*Sr6*, *Sr38*	0;	;2

* Infections types in seedling test: ; – chloroses, 0, 1, 1-2, 2 – resistant, 3, 4 – susceptible

** Avirulence/virulence on testing lines: *Sr11*, *Sr30*, *Sr9d*, *Sr24*, *Sr31 / Sr5*, *Sr21*, *Sr9e*, *Sr7b*, *Sr6*, *Sr8a*, *Sr9g*, *Sr36*, *Sr9b*, *Sr17*, *Sr9a*, *Sr10*, *SrTmp*, *Sr38*, *SrMcN*

*** Avirulence/virulence on testing lines: *Sr21*, *Sr11*, *Sr8a*, *Sr30*, *Sr24*, *Sr31 / Sr5*, *Sr9e*, *Sr7b*, *Sr6*, *Sr9g*, *Sr36*, *Sr9b*, *Sr17*, *Sr9a*, *Sr9d*, *Sr10*, *SrTmp*, *Sr38*, *SrMcN*

Considering the high frequency and importance of *Sr38* in local germplasm, we also compared field trial results of cultivars based on the presence of this gene to evaluate its effectiveness. The mean of field trial resistance score for cultivars with *Sr38* was 3.163 compared to 7.118 for cultivars without any Sr gene detected. Lowest disease severities were recorded for cultivars where *Sr38* was detected in a combination with one more Sr gene. The mean score was 2.094.

Table 12: Disease severity in studied wheat varieties in yearly field trials under artificial infection with Puccinia graminis f. sp tritici, on a scale of 1 to 9 (1: resistant; 9: susceptible). Second column lists genes detected using molecular markers

Cultivar/Year	Detected genes	2014	2015	2016	2017	2018	2019	2020	2021	2022
Angelus	none	3.5	-	-	-	-	-	-	-	-
Annie	*Sr38*	1.5	1.5	1.5	3.0	3.0	2.5	1.0	2.5	4.0
Apostel	none	-	-	-	-	9.0	9.0	-	-	-
Arkeos	none	-	-	-	-	6.0	-	-	-	-
Askaban	*Sr38*	-	-	-	-	3.5	3.0	3.0	-	-
Asory	*Sr24*, *Sr38*	-	-	-	-	-	2.5	1.0	1.0	2.0
Athlon	*Sr24*	4.0	4.0	-	-	-	-	-	-	-
Balitus	*Sr38*	2.0	3.0	3.0	3.0	4.0	4.0	1.0	6.5	4.0
Bernstein	*Sr38*	-	4.0	4.5	4.0	4.0	6.0	-	-	-
Bohemia	none	4.5	4.5	5.0	5.5	6.0	4.0	3.0	9.0	4.8
Bonanza	*Sr38*	-	3.0	3.0	3.0	-	-	-	-	1.0

Cultivar/Year	Detected genes	2014	2015	2016	2017	2018	2019	2020	2021	2022
Butterfly	*Sr38*	-	-	-	-	4.0	3.0	4.0	2.5	3.8
Campesino	*Sr24*, *Sr38*	-	-	-	-	-	1.0	1.0	1.0	3.5
Dagmar	*Sr38*	4.5	4.0	4.0	5.5	4.5	4.5	4.0	7.0	7.0
Elly	*Sr38*	4.0	3.0	3.5	4.0	-	4.0	4.0	-	3.0
Evina	*Sr38*	1.5	2.0	-	-	-	-	-	-	-
Fakir	none	4.0	4.0	6.0	8.0	8.0	7.5	3.5	7.5	6.5
Frisky	*Sr38*	-	2.0	2.0	2.5	3.0	3.5	1.0	3.0	2.5
Futurum	*Sr24*	-	-	1.0	2.0	2.5	4.0	2.0	-	-
Gaudio	*Sr38*	-	-	2.5	3.0	4.0	4.0	2.0	6.0	-
Genius	*Sr38*	4.0	2.5	3.5	4.0	3.0	3.0	2.5	3.0	3.0
Gordian	*Sr24*	-	7.5	8.0	7.5	7.5	8.0	8.0	-	-
Hyfi	*Sr24*	-	1.0	1.0	1.0	1.0	2.0	-	-	-
Illusion	*Sr38*	-	-	-	-	4.0	4.0	4.0	4.8	3.5
Johnson	*Sr31*	-	-	-	-	4.0	5.0	6.0	5.5	-
Julie	*Sr38*	2.0	-	2.0	3.5	3.0	4.0	3.5	3.0	3.5
LG Imposanto	*Sr38*	-	-	-	3.5	4.0	4.0	1.0	-	-
LG Mocca	none	-	-	-	-	9.0	9.0	9.0	8.5	6.5
Matchball	*Sr31*, *Sr38*	3.0	1.0	1.0	-	-	-	-	-	3.0
Patras	none	8.0	8.0	9.0	9.0	9.0	9.0	-	-	6.5
Penelope	*Sr38*	-	1.0	-	3.0	3.0	-	-	-	2.0
Pirueta	*Sr38*	-	-	-	-	3.5	3.5	3.5	3.0	4.3
Rebell	*Sr31*, *Sr38*	-	-	-	-	3.0	-	-	-	-
RGT Reform	*Sr38*	-	-	-	-	4.0	-	-	-	4.0
RGT Sacramento	*Sr38*	-	-	-	3.0	3.5	4.0	3.0	5.0	3.5
Rivero	*Sr38*	-	-	3.0	3.5	4.0	-	-	-	-
Sally	*Sr38*	-	-	-	-	-	4.0	4.0	4.8	-
Sheriff	*Sr24*	-	-	-	2.0	2.0	3.0	3.0	-	-
Steffi	*Sr38*	-	1.0	1.5	3.0	3.0	3.0	4.0	2.3	2.5
Tobak	none	9.0	9.0	9.0	9.0	-	-	-	-	-
Turandot	none	7.0	8.5	5.0	9.0	8.0	8.0	9.0	8.0	6.0
Vanessa	*Sr31*, *Sr38*	2.0	1.0	1.0	1.0	4.0	3.5	4.0	-	3.0
Viki	*Sr38*	-	-	2.5	4.0	-	-	-	-	-
Viriato	*Sr6*, *Sr38*	-	-	-	-	3.5	-	-	-	-

The 58 varieties were then tested for virulence to two races collected in 2020, TKRPF and PHRTF, to evaluate the effect of resistance genes that were postulated with molecular markers (Table 11). Those two races were chosen for the tests because their virulence profiles have been previously detected in collections from Czech Republic. The greenhouse tests confirmed that all cultivars with

Sr31 were resistant to both *Pgt* races. Cultivars with *Sr6* and *Sr24* were mostly resistant to the two races with some rare exceptions. Out of the 37 cultivars with *Sr38*, 17 were completely susceptible to the 2020 races, other eight cultivars were resistant only to race TKRPF which suggests presence of additional resistance in the cultivars.

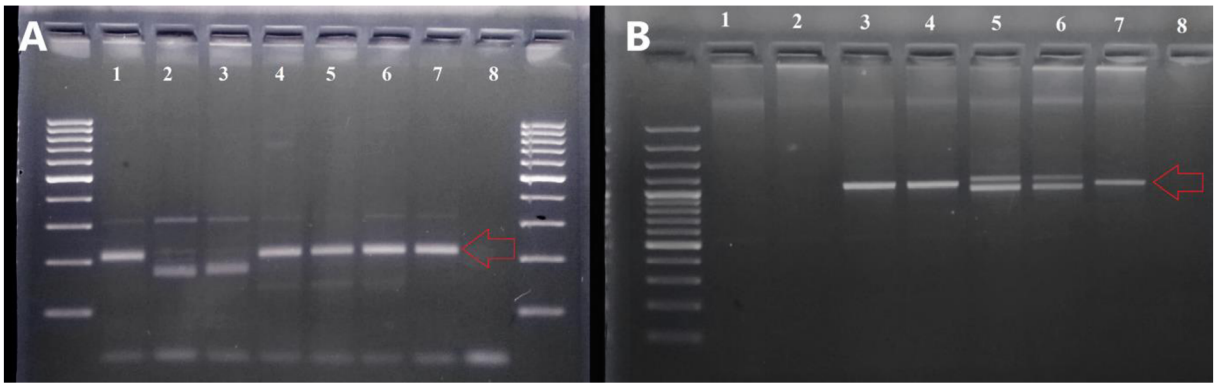

Figure 8: (A) Amplification of Sr6 marker Xcfd43 in 7 distinct wheat cultivars including Viriato. Arrow points to the resistant allele product of 215 bp. Lanes 2-3 are negative for Xcfd43. Lanes 4-7 are varieties used as positive controls. Lanes: 1, Viriato. 2, RGT Reform; 3, Apostel; 4, McMurachy; 5, Gatcher; 6, Shield; 7, Sr6 near isogenic line; 8, no template control (B) Amplification of the Sr31/Lr26 marker iag95 in various wheat cultivars visualized on a 1.6% agarose gel. Arrow points to the resistant allele. Lanes 3, 4, and 7 show the resistant allele. Lanes: 1, RGT Reform; 2, Julie; 3, Vanessa; 4, Johnson; 5, Asory; 6, Campesino; 7, Brigadier (positive control); 8, no template control. 100 bp DNA Ladder was loaded on the side lanes for comparison.

None of the tested resistance genes were detected in 11 cultivars in the panel of 58. The disease severities in those cultivars are corresponding with this finding both in the field and in the greenhouse. For instance, Tobak had a reported highest diseased severity in field trials in 2014 – 2017, Apostel performed the same way when tested in the field in 2018 and 2019 (Table 12). These results confirm that there are no effective major resistance genes in these cultivars. In case of Angelus, Bohemia, and Fakir, the results from field trials show a more moderate response, indicating presence of unknown genes. These genes could either be (i) catalogued major genes with

moderate effect that were not tested for in this study, (ii) novel, undescribed genes, or (iii) several QTLs conferring horizontal field resistance.

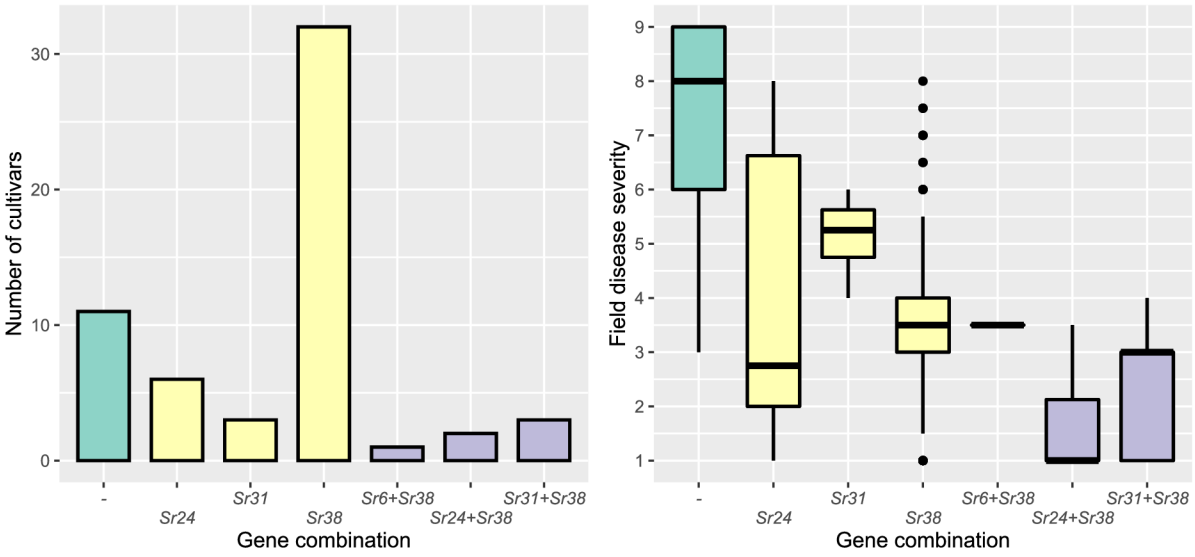

Figure 9: Gene combinations predicted by molecular marker assays. Left: Number of cultivars possessing each combination. Green color represents zero resistance genes, yellow color represents one resistance gene, purple represents a combination of two. Right: Recorded ratings from field trials for each gene combination (1 = no disease symptoms, 9 = maximum disease severity).

In greenhouse, several cultivars show unexplained resistant infection type with TKRPF in contrast with a susceptible PHRTF reaction. These are Evina, Julie, Penelope, Ponticus, Rivero, Steffi, Turandot, and Viki. TKRPF only had a contrasting avirulence to *Sr9d* which could be the cause if it is present in those cultivars, however this is unlikely. These cultivars must confer some race-specific resistance which is not part of the differential set. The greenhouse tests show further contrasting responses between the two races in Gordian, Hyfi, and RGT Reform, which were resistant to PHRTF but not TKRPF. This does not correspond with the marker results since Gordian and Hyfi showed positive results for the *Sr24* marker and both races should be avirulent to *Sr24*. Such discrepancy could be explained by false negative results of the marker assay. The avirulence to PHRTF in these three cultivars must be conferred by additional gene(s). This could be *Sr21* or *Sr8a*, which are part of the differential set. Presence of *Sr21* is highly unlikely since it was not used in commercial breeding. *Sr8a* could be present in our wheats. Unfortunately, the *Sr8a* marker assays used in this study did not give clear results. RGT Reform, however, shares the same alleles as control lines positive for *Sr8a* in all three KASP marker assays. Therefore, presence of *Sr8a* seems likely. To confirm this finding, it should be further tested with several races that differ in their virulence to this gene. Cultivars Arkeos and Chiron show resistant reactions to both the races. The genetic basis of this reaction is unknown.

KASP markers linked to *Sr15/Lr20/Pm1* and *Sr36* only yielded negative results in the panel as none of the 90 cultivars possessed the same allele as the samples used as positive controls. The results are not included in Table 13 for redundancy. Table 13 displays alleles of markers detected by KASP assays and includes results for cultivars used as positive controls. Marker wsnp_Ra_c3996_7334169 created 4 clusters in the allelic discrimination plot. 10.8% of the panel showed no amplification clustering together with the no template controls. 12.9% of cultivars were present in the cluster with both amplified alleles, 3.2% formed the G allele cluster and 73.1% formed the A allele cluster. The positive controls Asta, Tremie, and Hereford were all in the heterozygotic cluster. For the other two markers linked to *Sr8a*, there were no negative amplifications, and, in both cases, the positive controls amplified the A allele. For marker wsnp_Ku_c39334_47795461, 46.7% of the panel also amplified the A allele. For marker IWB22036, 63.3% amplified the A allele. The three *Sr8a*-linked markers show many contradictory results. Only nine cultivars possess the same allele combination as Asta, Tremie, and Hereford.

Two KASP markers reportedly linked to *Sr11* show contradictory results. Timstein was used as one of the cultivars possessing the gene and was scored as a heterozygote for both the markers. 6BL_IWB10724 clustered the samples into three groups. G allele, which was also amplified in the positive controls, was present in 65.6% of the cultivars. The rest of the panel either amplified the A allele or there was no amplification. In case of 6BL_IWB72471, only a single cultivar, Ponticus, possessed the same allele as the positive controls.

Table 13: Results of KASP marker assays linked to resistance genes to stem rust in a panel of 90 winter wheats cultivars.

	Markers linked to *Sr8a*			Markers linked to *Sr11*	
	wsnp_Ra_c3996_7334169	**wsnp_Ku_c39334_47795461**	**IWB22036 (kwh54)**	**6BL_IWB10724**	**6BL_IWB72471**
LG Mocca	A	G	G	G	A
Campesino	A	G	G	No amplification	A
Rivero	A	G	G	A	A
Arkeos	A	G	Heterozygote	No amplification	A
RGT Reform	Heterozygote	Heterozygote	Heterozygote	G	A
Evina	A	G	G	G	A
Julie	A	G	Heterozygote	G	A
Viriato	Heterozygote	Heterozygote	Heterozygote	G	A
Ponticus	A	G	Heterozygote	A	C
Frisky	A	G	G	G	A
Johnson	A	G	Heterozygote	G	A
Fenomen	A	G	G	G	A
Dagmar	A	G	G	G	A
Avenue	No amplification	Heterozygote	Heterozygote	No amplification	A

	Markers linked to *Sr8a*			Markers linked to *Sr11*	
	wsnp_Ra_c3996_7334169	**wsnp_Ku_c39334_47795461**	**IWB22036 (kwh54)**	**6BL_IWB10724**	**6BL_IWB72471**
Genius	A	Heterozygote	Heterozygote	G	A
RGT Sacramento	A	G	G	G	A
Turandot	A	G	Heterozygote	G	A
Illusion	A	A	Heterozygote	G	A
Bonanza	A	A	Heterozygote	No amplification	A
Vanessa	A	Heterozygote	Heterozygote	G	A
Sofru	A	G	G	G	A
Butterfly	A	Heterozygote	Heterozygote	G	A
Pirueta	Heterozygote	Heterozygote	Heterozygote	G	A
Fakir	A	Heterozygote	Heterozygote	A	A
Patras	A	Heterozygote	Heterozygote	G	A
Megan	A	G	G	G	A
KWS Elementary	G	Heterozygote	Heterozygote	G	A
LG Orlice	A	G	G	G	A
Asory	A	G	Heterozygote	G	A
LG Absalon	Heterozygote	Heterozygote	Heterozygote	G	A
Tobak	A	G	G	A	A
LG Keramik	No amplification	Heterozygote	Heterozygote	G	A
Bodyček	A	G	G	G	A
Sheriff	No amplification	G	G	G	A
LG Imposanto	No amplification	Heterozygote	Heterozygote	G	A
Chiron	A	G	G	No amplification	A
Steffi	Heterozygote	Heterozygote	Heterozygote	A	A
Balitus	A	Heterozygote	Heterozygote	No amplification	A
Bohemia	A	Heterozygote	Heterozygote	No amplification	A
Askaban	A	G	G	No amplification	A
Grizzly	A	G	Heterozygote	G	A
Advokat	A	Heterozygote	Heterozygote	A	A
LG Dita	A	G	G	G	A
Elixer	A	G	G	G	A
Gordian	No amplification	Heterozygote	Heterozygote	A	A
Rebell	A	Heterozygote	Heterozygote	G	A
Futurum	A	Heterozygote	Heterozygote	G	A
Brilliant	A	G	G	A	A
Annie	A	G	Heterozygote	A	A
Kalbex	A	G	Heterozygote	G	A
Judita	A	G	Heterozygote	A	A
Matchball	G	Heterozygote	Heterozygote	G	A
Pannonia NS	A	Heterozygote	Heterozygote	A	A
Athlon	Heterozygote	Heterozygote	Heterozygote	G	A

	Markers linked to *Sr8a*			Markers linked to *Sr11*	
	wsnp_Ra_c3996_7334169	**wsnp_Ku_c39334_47795461**	**IWB22036 (kwh54)**	**6BL_IWB10724**	**6BL_IWB72471**
Golem	No amplification	Heterozygote	Heterozygote	G	A
Lear	A	G	G	G	A
Penelope	A	G	Heterozygote	G	A
Elly	A	G	G	No amplification	A
Viki	A	G	Heterozygote	G	A
Nelson	A	Heterozygote	Heterozygote	No amplification	A
JB Asano	A	G	Heterozygote	G	A
Ostroga	Heterozygote	Heterozygote	Heterozygote	No amplification	A
Nordika	A	G	G	A	A
Bardotka	A	Heterozygote	Heterozygote	No amplification	A
LG Mondial	A	G	G	G	A
Skif	A	G	Heterozygote	G	A
KWS Donovan	A	G	G	G	A
Adina	A	G	G	G	A
Crossway	A	G	G	G	A
Mercedes	A	G	G	G	A
WPB Calgary	Heterozygote	Heterozygote	Heterozygote	G	A
RGT Premiant	A	G	G	G	A
Partner	A	G	Heterozygote	G	A
Proteus	Heterozygote	Heterozygote	Heterozygote	G	A
SU Tarroca	No amplification	Heterozygote	Heterozygote	G	A
Potenzial	A	Heterozygote	Heterozygote	G	A
Cimrmanova raná	A	Heterozygote	Heterozygote	G	A
Papageno	A	G	G	No amplification	A
Mulan	A	G	Heterozygote	G	A
Magister	A	Heterozygote	G	No amplification	A
Arktis	No amplification	Heterozygote	Heterozygote	No amplification	A
Federer	A	G	G	A	A
Forhand	No amplification	Heterozygote	Heterozygote	G	A
Akteur	No amplification	Heterozygote	Heterozygote	G	A
Seladon	A	Heterozygote	Heterozygote	No amplification	A
Sailor	A	Heterozygote	G	No amplification	A
Baletka	A	G	Heterozygote	G	A
Tiguan	G	Heterozygote	Heterozygote	G	A
Etana	A	G	G	G	A
Diadem	A	Heterozygote	Heterozygote	A	A
Asta (Sr8a)	Heterozygote	Heterozygote	Heterozygote	-	-
Tremie (Sr8a)	Heterozygote	Heterozygote	Heterozygote	-	-
Hereford (Sr8a)	Heterozygote	Heterozygote	Heterozygote	-	-

	Markers linked to *Sr8a*			Markers linked to *Sr11*	
	wsnp_Ra_c3996_7334169	**wsnp_Ku_c39334_47795461**	**IWB22036 (kwh54)**	**6BL_IWB10724**	**6BL_IWB72471**
Kenya Farmer (Sr11)	-	-	-	G	C
Timstein (Sr11)	-	-	-	Heterozygote	Heterozygote
Trident (Sr11)	-	-	-	G	C

6.2. Seedling and adult plant phenotyping

Infection type in seven lines infected with RCHPC and TRTTF was scored at the seedling stage. This was done in five individual repetitions (single plants). The most representative phenotype for each treatment is shown in Table 15. One Michigan Amber - TRTTF sample did not reach the second leaf stage and the data are therefore missing. Arkeos and Campesino were highly resistant to both races with ratings ranging from 0 to ;1. Evina and RGT Reform were rated as resistant for RCHPC but susceptible (3) for TRTTF. Rivero, LG Mocca and Michigan Amber were all rated from 3 to 4 as fully susceptible.

Table 14: Growth stage for plants prior to inoculation in a cultivation chamber, grouped by cultivar.

Cultivar	Growth stage, mean	Standard error
Arkeos	62.2	0.51
Campesino	58.7	0.42
Evina	41.1	0.31
LG Mocca	38.3	1.05
Rivero	44.9	2.05
RGT Reform	44.1	2.33
M-A	56.1	3.06

The plants for adult plant experiments were successfully vernalized and reached adult stage, however, the growth speed differed between plants and between cultivars, therefore, growth stage was recorded for each plant prior to inoculation according using a decimal scale (Table 14). The growth stage varied from booting to flowering stages with Arkeos being the earliest cultivar. Flag leaf was fully developed in all samples prior to inoculation.

Disease severity was rated using a numerical scale separately for bulked flag leaves and the leaves below flag leaves and data are presented in Table 15. All treatments of Arkeos, Campesino, and Rivero were rated 1.0 as they showed no disease symptoms. Michigan Amber, which was used as a universally susceptible positive control, shower higher disease severity for TRTTF; 8.5 for flag leaf and 8.0 for the leaf below flag leaf. RGT Reform was without disease symptoms for RCHPC

but 4/4.5 for TRTTF. LG Mocca recorded the highest disease severity of the tested cultivars, with the maximum rating on flag leaf infected with TRTTF.

6.3. Image analysis and fungal tissue quantification

A single classifier was successfully trained for both the seedling and adult plant leaves in Trainable Weka Segmentation. It categorized pixels in the scanned leaf images into pustule, chlorosis, leaf, and background classes. Since the data from image analysis were generated by a single classifier, the values for both seedlings and adult plants are presented in a single table (Table 15). The classifier quantified the area of leaf covered by pustules, ranging from 0.00% in cultivars rated as completely resistant to 16.60% for LG Mocca seedling infected with race RCHPC. By visual assessment, that reaction was rated a 4 on the Stakman scale. The coverage of leaves by chlorosis ranged from 0.02% in a reaction that was rated from immune to 24.32% in a heavily infected seedling of LG Mocca. The ratio between leaf area cover by pustules and chlorosis was calculated for all samples. This ratio only exceeded 1.0 in adult plants of the most susceptible cultivars LG Mocca and Michigan Amber.

Table 15: Image analysis of disease symptoms on infected leaves of seven wheat cultivars. Results for two races of Puccinia graminis f. sp. tritici are shown separately. Disease symptom severities are represented by percentage of leaf area covered by pustules and chlorosis. Data from visual assessment is included for comparison using appropriate scales.

Cultivar	Race	Leaf	Pustules	Chlorosis	Pustules / Chlorosis	Infection type*	Disease severity**
Arkeos	**RCHPC**	Flag	0.28%	0.94%	0.29	-	4
		Flag-1	0.04%	0.68%	0.06	-	3.5
		Seedling	0.01%	0.27%	0.05	;2	-
	TRTTF	Flag	0.31%	2.89%	0.11	-	6
		Flag-1	0.35%	4.79%	0.07	-	5
		Seedling	0.09%	0.87%	0.11	;2	-
Campesino	**RCHPC**	Flag	0.01%	0.19%	0.06	-	1
		Flag-1	0.02%	0.27%	0.06	-	1
		Seedling	0.04%	1.12%	0.03	;1	-
	TRTTF	Flag	0.01%	0.02%	0.42	-	1
		Flag-1	0.01%	0.18%	0.06	-	1
		Seedling	0.23%	3.57%	0.06	;	-
Evina	**RCHPC**	Flag	0.01%	0.38%	0.04	-	1
		Flag-1	0.00%	0.52%	0.00	-	1
		Seedling	0.07%	1.81%	0.04	;2	-
	TRTTF	Flag	0.02%	0.63%	0.03	-	1
		Flag-1	0.06%	1.56%	0.04	-	1
		Seedling	4.12%	14.83%	0.28	3	-
LG Mocca	**RCHPC**	Flag	3.11%	2.54%	1.22	-	7
		Flag-1	1.87%	1.77%	1.06	-	6.5

		Seedling	16.60%	22.19%	0.75	4	-
	TRTTF	Flag	12.57%	9.19%	1.37	-	9
		Flag-1	5.94%	3.82%	1.56	-	7.5
		Seedling	13.13%	24.32%	0.54	4	-
Rivero	**RCHPC**	Flag	0.00%	0.07%	0.02	-	1
		Flag-1	0.00%	0.02%	0.00	-	1
		Seedling	1.91%	6.23%	0.31	3	-
	TRTTF	Flag	0.00%	0.05%	0.03	-	1
		Flag-1	0.01%	0.04%	0.17	-	1
		Seedling	6.92%	19.15%	0.36	4	-
RGT Reform	**RCHPC**	Flag	0.00%	0.05%	0.03	-	1
		Flag-1	0.08%	0.53%	0.16	-	1
		Seedling	0.03%	0.08%	0.46	;	-
	TRTTF	Flag	0.09%	0.13%	0.71	-	4
		Flag-1	0.31%	0.30%	1.04	-	4.5
		Seedling	0.03%	0.51%	0.05	3	-
Michigan Amber	**RCHPC**	Flag	3.59%	2.16%	1.66	-	7
		Flag-1	1.48%	1.32%	1.12	-	6.5
		Seedling	7.89%	18.42%	0.43	4	-
	TRTTF	Flag	6.81%	3.28%	2.07	-	8.5
		Flag-1	5.85%	4.54%	1.29	-	8
		Seedling	4.87%	13.76%	0.35	4	-

*** Infections types in seedling test: ; – chloroses, 0, 1, 1-2, 2 – resistant, 3, 4 – susceptible (see Stakman et al. 1962 for original scale)**
**** On a numerical scale from 1-9 where 1 is absence of symptoms and 9 is a maximum disease severity (Miedaner, 2016)**

Seedling leaves, adult flag leaves and adult leaves below flag leaves for each treatment were used for WAC after scanning them for image analysis. The leaves were ground, and fungal tissue was quantified in the samples. Reads of relative fluorescent units (RFU, Figure 10) were used to fit linear mixed models. Post-hoc statistical tests were performed to compare RFUs depending on cultivar. After establishing significant differences between pathogen races, they were compared separately. 95% confidence intervals for all pairwise comparisons are shown in Figure 11. In seedling infections with RCHPC, LG Mocca is not significantly different from the susceptible control Michigan Amber, together they recorded the highest amount of chitin. In case of TRTTF, LG Mocca surpassed the positive control and shows the highest infection. In RCHPC infections, Campesino, Evina, and RGT Reform formed a group of the least infected cultivars, not significantly different between each other. Arkeos and Campesino had the similar, lowest, ratings with TRTTF. This demonstrates clear differences between the physiological races and seedling resistance in the cultivars.

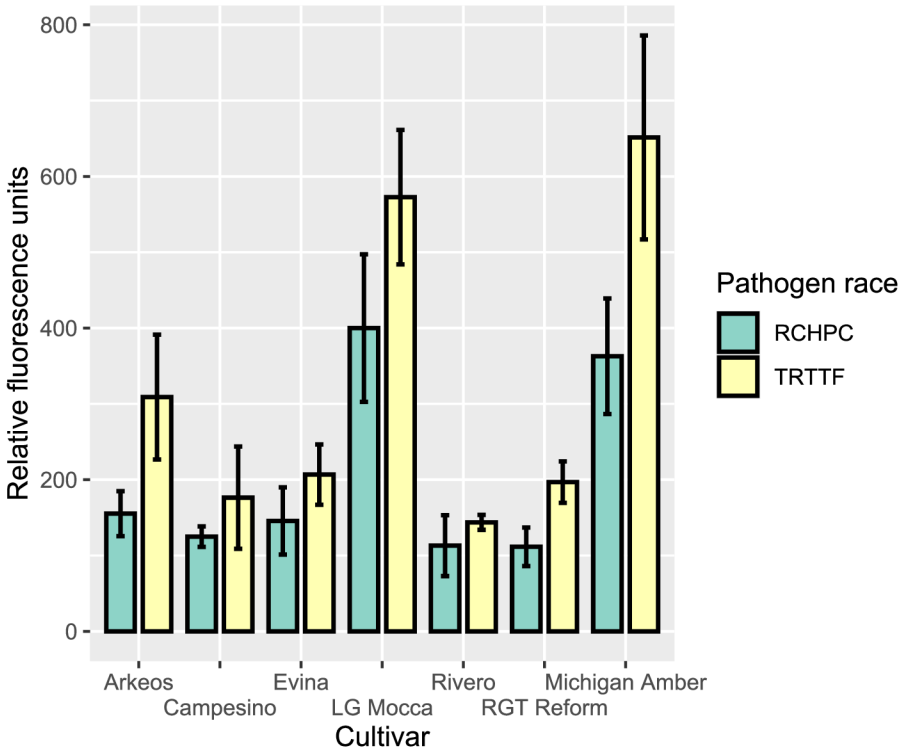

Figure 10: Bar plot showing measured fluorescence of WGA-FITC bound to fungal chitin in infected adult plant leaf samples. Error bars show standard deviation of measured values. This graph groups data for both flag and flag-1 leaves and shows the mean values.

In case of RCHPC infections in adult stage, the cultivars could be separated in two groups. First group consists of five completely resistant cultivars Arkeos, Campesino, Evina, Rivero, and RGT Reform. None of the pairwise comparisons between these showed a significant statistical difference. The second group is LG Mocca and Michigan Amber which did not differ significantly. In case of TRTTF on adult plants, Arkeos samples have higher RFU compared to Campesino and the other resistant varieties. Furthermore, Michigan Amber, the susceptible control, shows slighly higher infection compared to LG Mocca.

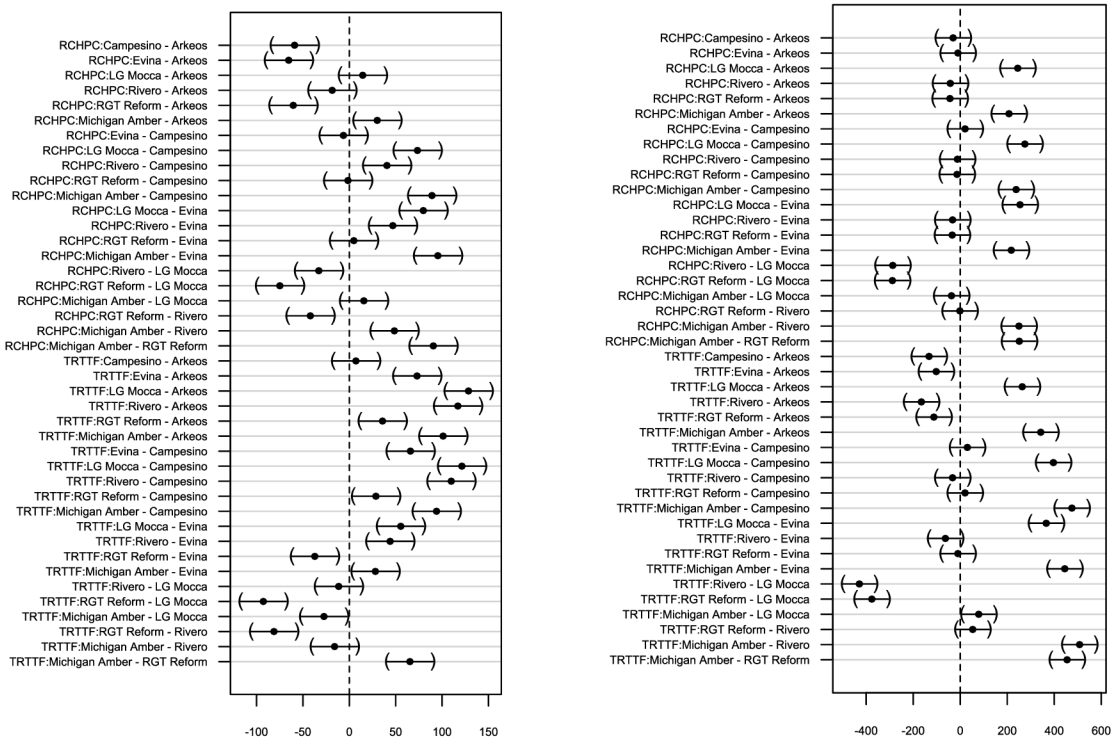

Figure 11: 95% family-wise confidence intervals for pairwise comparisons between cultivars. Projected values of relative fluoresence units from a WAC assay are based on a linear mixed model fitted with lmer. Left graph shows results for seedling tests and right side shows results for adult plants. RCHPC and TRTTF races are shown separately.

6.4. Marker assays in the spelt wheat panel

Out of 6 molecular markers indicating possible presence of a stem rust resistance gene, only one marker was positive in a single case. Marker iag95 showed a positive result for Black Forest spelt wheat. The rest of the results were negative (Table 16). Table 16 also shows results for leaf rust resistance gene markers as the relevant study was covering leaf rust as well. The markers linked to *Lr1* and *Lr10* show majority of positive results in the panel of 16 spelt wheats.

Table 16: Spelt wheat cultivars and the results of molecular marker assays indicating a possible presence of a corresponding resistance gene. Positive result of a marker assay is marked by a plus sign.

Cultivar	*Lr1*	*Lr9*	*Lr10*	*Lr19 Sr25*	*Lr24 Sr24*	*Lr26 Sr31 Yr9*	*Lr28*	*Lr34 Sr57 Yr18*	*Lr37 Sr38 Yr17*	Sr6
Badenkrone	+	-	-	-	-	-	-	-	-	-
Black Forest	-	-	+	-	-	+	-	-	-	-
Epanis	+	-	+	-	-	-	-	-	-	-
Farnsburger Rotkorn (Fb6)	+	-	+	-	-	-	-	-	-	-
Filderstolz	-	-	+	-	-	-	-	-	-	-
Fuggers Babenhauser Zuchtveesen	+	-	+	-	-	-	-	-	-	-
Gugg 4H	+	-	+	-	-	-	-	-	-	-
Öko 10	+	-	+	-	-	-	-	-	-	-
Rottweiler Dinkel St. 6	-	-	+	-	-	-	-	-	-	-
Rottweiler Frühkorn	+	-	-	-	-	-	-	-	-	-
Samir	+	-	-	-	-	-	-	-	-	-
Sofia 1	+	-	-	-	-	-	-	-	-	-
Speltvete fran Gotland	-	-	+	-	-	-	-	-	-	-
Spy	+	-	-	-	-	-	-	-	-	-
von Rechbergs Früher Winterspelz	+	-	-	-	-	-	-	-	-	-
Zürcher Oberländer Rotkorn	+	-	+	-	-	-	-	-	-	-

7. Discussion

7.1. Resistance gene composition in tested cultivars

Our results demonstrate that wheat cultivars in Czech Republic rely heavily on *Sr38*, its frequency is higher than that reported by Flath et al. (2018) in cultivars grown in Germany. Cultivars with this gene still showed higher average resistance in yearly non-race-specific field trials (Table 12, Figure 9). However, greenhouse tests with two isolates collected in 2020 show that resistance conferred by *Sr38* is overcome by new *Pgt* races. This dependency on a single R gene can cause problems when such new races spread, because virulent races gain evolutionary advantage and can therefore spread quicker. Several races of *Pgt* caused the disease outbreak in Germany in 2013. They were later described (Olivera Firpo et al. 2017) and all of them were virulent to wheat cultivars with *Sr38* gene. Similarly, a Rust Tracker tool created by Global Rust Reference Center (https://rusttracker.cimmyt.org/?page_id=6647) reports races TTRTF and TTKTF in recent samples from Slovakia and Austria that both overcome the *Sr38* resistance.

Sr31 is a gene that should be universally effective against stem rust on the European continent, as the Ug99 group races are not currently present (Patpour et al. 2022a). The combination of *Sr31* with *Sr38* was detected in 3 cultivars. The field trial ratings for Vanessa, Rebell, and Matchball from various years ranged between 1.0 to 4.0. Combination of *Sr24* and *Sr38* was postulated in cultivar Campesino which was rated fully immune in years 2019 – 2021 but recorded minor disease symptoms in 2022. This could possibly be due to higher disease pressure or new appearance of more virulent races.

Markers associated with genes *Sr2*, *Sr15*, *Sr25*, and *Sr36* showed only negative results in our selections. *Sr2* has been used extensively, but primarily by CIMMYT for wheat growing regions of South America, Africa, and Asia. Indeed, at points in 20th century, this gene was present in more than 90% of CIMMYT germplasm because of its durability (Rajaram et al. 1988). It was however mostly used in a complex with other resistance genes. The gene was apparently not introduced to European winter wheats. It is associated with a phenotypic trait of pseudo black chaff (Tabe et al. 2019), which has not been observed during our field trials. Similarly, *Sr15*/*Lr20*/*Pm1* has not been detected in our selections using either STS or KASP marker. This gene is possibly only present in spring wheat and would have to be used in combination with other genes to provide good defence. *Sr36* has been used in European breeding historically, notably in cultivar Agra, but also in Croatian and Hungarian wheats (Purnhauser et al. 2011). Our results indicate that wheat cultivars recently grown in the Czech Republic do not contain *Sr36* and it would not be effective against currently important Pgt races (notably TKTTF and TTRTF).

The presence of major resistance genes can be related to the stem rust races that currently dominate in the population and their virulence. The most widely detected gene in our study has been effective against the indigenous European races of clade VIII such as RSBNC, RGHPC, RFCNC, RFCPC (Patpour et al. 2022a; Szabo et al. 2022) and many other variations. *Sr38* is, however, of no use towards the most prominent virulent races of today. Both Sicily race (TTRTF) and the Digalu race (TKTTF) are virulent to cultivars with this gene (Patpour et al. 2022a). PKPTF, TKKTF, TKKTP, all from the IV clade, previously detected in Germany in 2013, overcome the *Sr38* resistance gene. Any of the Ug99 lineage races detected so far (TTKSK, TTKST, TTTSK, TTHST, TTKTK, TTKTT, TTKSF, TTKSP, PTKST, TTKSF+) would also render this gene inefficient (Visser et al. 2009; Newcomb et al. 2016), if they crossed the Mediterranean to Europe. The situation with *Sr24* and *Sr31* is slightly more optimistic. These genes should be efficient against the Sicily race and Digalu race currently dominating the European continent. TKKTP detected in Germany previously (Olivera Firpo et al. 2017; Guo et al. 2022) would break down the *Sr24* resistance. Most of the Ug99 lineage races would break down *Sr31* resistance and some of the recent ones also *Sr24* resistance if they were not restrained to Africa and Middle Asia. Unfortunately, this study was not able to establish the presence of *Sr11* using the two KASP markers. *Sr11* would be effective towards TKTTF, PKPTF, and TKKTF, some of the prominent races in Europe.

Table 17 is an overview of wheat stem rust resistance genes that were catalogued up to the point of writing this thesis and provides short information of current use of each of the genes in wheats grown in Czech Republic. This table puts findings of this study into perspective of current knowledge. It is apparent that many of the catalogued genes were introgressed into hexaploid wheat from related species but are only present in resulting experimental lines. Often such genes have not been deployed in commercial cultivars, thus their presence in our wheats is described as highly unlikely. Linkage drag can be a major reason for not introducing the segments in elite cultivars. The introduced segment carrying the gene needs to be reduced as much as possible and only after that the stocks are more suitable for breeding programs. Such efforts have been undertaken with genes such as *Sr22* (Olson et al. 2010a), *Sr39* (Mago et al. 2009), or *Sr53* (Liu et al. 2011b). Some of the catalogued genes have been previously reported in European cultivars, such as *Sr29* (McIntosh et al. 1995; Pathan & Park 2007), which was not analyzed in this study due to absence of linked molecular markers. These genes are not part of the North American differential set and thus even the virulence of collected pathogen samples to this gene is not known. It would be interesting to include cultivars that carry *Sr29* into a differential set and see if they differentiate local pathogen races. Such cultivars could be Etoile de Choisy, Mara and Moisson (McIntosh et al. 1995) or set of cultivars that most probably possess the gene such as Bruta, Orqual, Sarka, Scipion, Texel, and

Thesee (Pathan & Park 2007). Furthermore, several of the genes in the overview are not considered useful anymore since virulent races are widely present and therefore their effect is negligible.

Table 17: A summary of catalogued wheat stem rust resistance genes and their current status of use in Czech Republic. Basic information about genes up to Sr40 can be found in McIntosh et al. (1995) and further sources are included only when necessary.

Resistance gene	Description	Status of use in Czech Republic
Sr1	Abandoned designation	-
Sr2	Partial resistance, APR, associated with pseudo-black chaff, widely deployed in CIMMYT spring wheats, non-race specific	Not found in winter wheat cultivars in Czech Republic using a molecular marker (this study). Associated PBC trait not reported.
Sr3-4	Abandoned designation	-
Sr5	"Immunity gene". Very low infection types until overcome by virulent races.	Deployed in Czech Republic, now obsolete due to virulence in present races (Bartoš et al. 1970; Bartoš 1975b).
Sr6	Temperature and light-sensitive, race-specific with somewhat variable resistant phenotype. Present in African, American, Australian cultivars. Largely overcome.	Postulated in one cultivar by a molecular marker (this study). Probably has no effect due to widely present virulence.
Sr7a, b	Not consciously introgressed. Present in many wheats worldwide. Virulent isolates present in all wheat growing areas.	Unknown status. Ineffective gene.
Sr8a	Wheat allele of *Sr8*. Mild effect, not sufficient under high disease pressure. Worldwide use.	Was deployed in local cultivars but avirulence is now rare. Markers gave conflicting results (this study).
Sr9a, 9b	Virulence present everywhere. Not important in breeding.	Unknown status. Ineffective.
Sr9c	Abandoned designation	-
Sr9d	Previously *Sr1*, linked with *Lr13*. No importance in agriculture.	Unknown status. Ineffective.
Sr9e, 9f, 9g	No importance in agriculture	Unknown status. Ineffective.
Sr10	Reported to be common in some CIMMYT materials. Now obsolete.	No markers available. Ineffective against local races.
Sr11	Worldwide use but virulence present in all regions.	Postulated presence in some old cultivars. Virulence in local races common. Markers tested in this study without clear results (this study).
Sr12	Temperature-sensitive gene from durum wheat. Possibly quite widespread but virulence is common.	Unknown status.
Sr13	Possibly present in some durum wheats. Experimentally transferred to hexaploid wheat.	Unknown. Highly unlikely to be present.
Sr14	Rarely used in commercial wheats.	Unknown. Highly unlikely to be present.
Sr15	Low-temperature dependent, linked to *Lr20*	Not detected using a KASP marker (this study).
Sr16	Gene with low effect. Possibly present in some commercial wheats but not reported due to virulence of isolates. Not deployed in breeding intentionally.	Unknown status. Ineffective gene.
Sr17	Temperature-sensitive. Linked with *Pm5* and *Lr14a*.	Unknown frequency. Ineffective against local races.
Sr18	Rarely identified even though it is possibly common. Low effect. No use in agriculture.	Unknown status. Ineffective gene.
Sr19-20	Rarely identified. Mostly ineffective.	Unknown status. Ineffective gene.
Sr21	Gene from diploid wheats. High-temperature dependent. Not used in commercial hexaploid wheats.	Unknown. Highly unlikely to be present. Virulent isolates frequent.
Sr22	Temperature-sensitive. Very limited deployment in commercial wheats so far.	Unknown. Highly unlikely to be present.
Sr23	Completely linked to *Lr16*. Requires high temperature and light. No use in agriculture.	Unknown status. Ineffective gene.

Resistance gene	Description	Status of use in Czech Republic
Sr24	On an *Aegliops elongatum* translocation together with *Lr24*. Worldwide use, overcome by some Ug99 lineage races.	Used in Czech Republic. Effective against local races (this study).
Sr25	Backcrossed into Australian wheat from *Thinopyrum ponticum*. Linked with *Lr19*. Cultivars characterized by yellow endosperm pigment.	Not found in Czech cultivars using a molecular marker (this study). Associated trait not observed.
Sr26	Only use in commercial cultivar breeding reported in Australia.	Unknown. Highly unlikely to be present.
Sr27	From triticale. Not used in commercial wheat.	Unknown. Highly unlikely to be present.
Sr28	Limited use in breeding, only reported in few North American lines.	Unknown. Highly unlikely to be present.
Sr29	Possibly a gene of European origin. Previously designated *SrEC*.	Reported to be present and effective in Czechoslovakian cultivars (Bartoš et al. 1993, 1994b, 1996). Current frequency unknown. Markers not available.
Sr30	From cultivar Webster (Knott & McIntosh 1978). Common in spring wheat landraces worldwide, a gene with somewhat variable response. Virulence common everywhere (Bansal et al. 2008; Kankwatsa et al. 2017; Shamanin et al. 2020).	Unknown status. Virulent races common. Markers not available.
Sr31	On rye translocation or substitution together with Lr26. Mostly present on a 1BL.1RS translocation from Pektus rye.	Widely used in breeding, cultivars with translocation still grown in Czech Republic. So far remains effective against local races (this study).
Sr32	Introgressions from *Aegilops speltoides* (Mago et al. 2013). No reported use in commercial breeding.	Unknown. Highly unlikely to be present.
Sr33	Introgression from *Aegilops tauschii*. No reported use in commercial breeding.	Unknown. Highly unlikely to be present.
Sr34	Introgression from *Aegilops comosa*. No reported use in commercial breeding.	Unknown. Highly unlikely to be present.
Sr35	Introgression from *T. monococcum*	Unknown. Highly unlikely to be present.
Sr36	From *T. timopheevii*. Has been used widely in 20th century and was reported in European cultivars too.	Not used anymore. Overcome by new races. Not found in current cultivars (this study).
Sr37	From *T. timopheevii*. Not used in commercial breeding due to linkage drag.	Unknown. Highly unlikely to be present.
Sr38	*Lr37-Yr17-Sr38* complex. Deployed worldwide.	Widely deployed in Czech Republic, but virulent races are now present (this study).
Sr39–40	No reported use in commercial cultivars.	Unknown. Highly unlikely to be present.
Sr41	Detected in spring wheat Waldron (Riede et al. 1995). No known use in breeding.	Unknown. Highly unlikely to be present.
Sr42/SrTmp/SrCad	Closely related genes, perhaps alleles of the same genes or linked genes (Kassa et al. 2016; Hiebert et al. 2016).	Unknown. *SrTmp* was reported before in Czech cultivars (Bartoš et al. 2002). Robust markers missing.
Sr43–47	Introgressions from related species (Liu et al. 2013; Niu et al. 2014; Periyannan et al. 2014; Yu et al. 2015), not yet deployed in commercial cultivars.	Unknown. Highly unlikely to be present.
Sr48	Gene from cultivar Arina (Nsabiyera et al. 2023), reported to be present in other European cultivars (Pathan & Park 2007).	Unknown, possibly present. Marker not tested.
Sr49	Identified recently in a Tunisian landrace (Bansal et al. 2015)	Unknown. Highly unlikely to be present.
Sr50	Comes from the same chromosome arm of rye as *Sr31* but is present in different translocations (Mago et al. 2002). Use in breeding not reported.	Unknown. Highly unlikely to be present. Could be tested with an available marker.
Sr51–53	Recent introgressions from related species (Liu et al. 2011a, 2011b; Qi et al. 2011), no reported use in commercial cultivars.	Unknown. Highly unlikely to be present.
Sr54	Identified recently in Norin 40, no reported use in breeding (Ghazvini et al. 2013).	Unknown.

Resistance gene	Description	Status of use in Czech Republic
Sr55–58	APR genes used in CIMMYT breeding, used for gene-pyramiding, non-race-specific resistance (Kerber & Aung 1999; Herrera-Foessel et al. 2014b; Kolmer et al. 2015).	Presence not reported in Czech cultivars and unlikely. Associated leaf-tip necrosis trait not known in Czech cultivars.
Sr59–62	Recent introgressions, no reported use in commercial cultivars (Rahmatov et al. 2016; Chen et al. 2018a; Zhang et al. 2021; Yu et al. 2022).	Unknown. Highly unlikely to be present.
Sr63	APR in durum wheat (Mago et al. 2022).	Unknown. Highly unlikely to be present.

7.2. Molecular marker validation

PCR-based markers have been a part of wheat breeding for decades now, even though the hexaploid wheat genome is large and consists mostly of repetitive regions (Paux et al. 2012). In MAS, the power of markers lies in speeding up the process of selection. They can also be used to postulate or confirm the presence of resistance genes in commercial cultivars. However, the results are easy to misinterpret, and accuracy of markers depends on many factors. For instance, transferability of markers from one genetic background to another can be an issue, and markers should therefore be tested on groups of cultivars from different areas (Miedaner & Korzun 2012). Ideally, molecular markers are directly converted from polymorphic sequences within the genes, and work in any genetic background of wheat. Such markers are called perfect, diagnostic, or functional (Bagge et al. 2007). With many available markers, this is not the case. The markers discussed in chapter 7.1. (listed in Table 7) have been validated in multiple genetic backgrounds and have each been reported in several publications. Additionally, the results were then compared with phenotypic tests and discussed together. This is, however, not the case with recently published KASP markers linked to *Sr8a* and *Sr11*, which have not yet been validated in this genetic background. These will be discussed in the following paragraphs.

The increasing number of reported markers indicate that KASP is becoming a standard technology for wheat markers (Rasheed et al. 2016; Kaur et al. 2020). Here, basic groundwork for validating three SNP markers linked to *Sr8a* and two markers linked to *Sr11* was established in a genetic background of European winter wheat. In case of *Sr8a*, nine cultivars share the same three SNP alleles as the *Sr8a*-positive reference lines Asta, Tremie, and Hereford (Table 13). However, there is a lot of recombination between the loci. Furthermore, in case of the first used marker, the positive lines cluster in the heterozygotic group (Figure 12). This can be caused by erroneous primer design causing homologous alleles from the other two wheat genomes to amplify, which is a common issue in hexaploid wheat (Makhoul et al. 2020; Maccaferri et al. 2022). Next steps in validating the markers should be phenotypic tests of the lines with stem rust races with contrasting virulence to the gene.

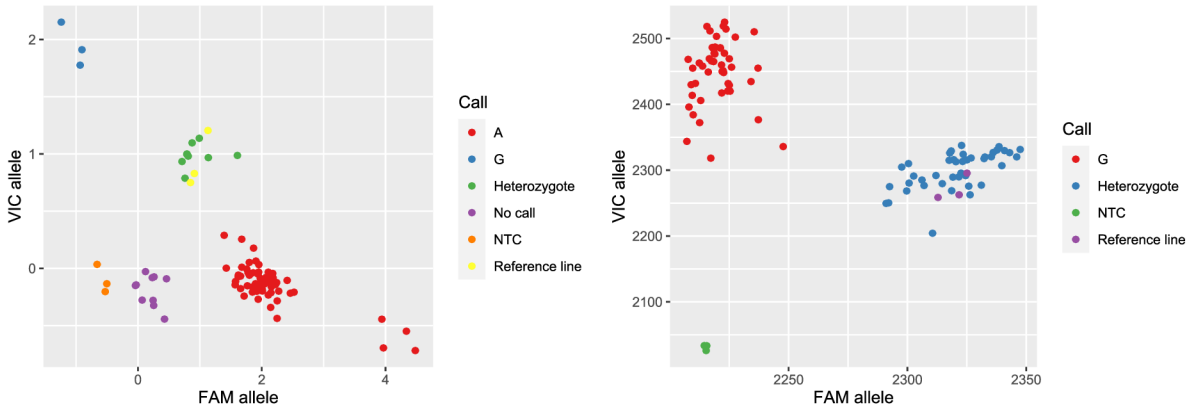

Figure 12: Allelic discrimination plots for KASP markers wsnp_Ra_c3996_7334169 (left) and wsnp_Ku_c39334_47795461 (right). The 96 samples consist of a panel of 90 winter wheats, three reference lines with a reported presence of Sr8a gene, and three wells with no DNA (NTC stands for no template control). Fluorescence is measured in relative fluorescence units (RFU).

In case of *Sr11* markers reported by Nirmala (2016), three lines were used as references, out of which Timstein amplifies both the alleles in both markers. This is possibly due to contamination of the sample, or the line is not fully homozygous. One of the markers puts a single cultivar, Ponticus, together with Kenya Farmer and Trident. The other marker shows variable results, including a cluster of no amplification in the locus (Figure 13). Please note that the original study (Nirmala et al. 2016) reports results of the marker assays using the complementary primer nucleotides, while in this study the actual polymorphisms in wheat are shown. In next steps, it would be interesting to do phenotypic tests with Ponticus and several lines showing positive results for the 6BL_IWB10724 marker and find out if *Sr11* resistance is linked with either. Pathogen isolates polymorphic in virulence to the gene should be used for these experiments.

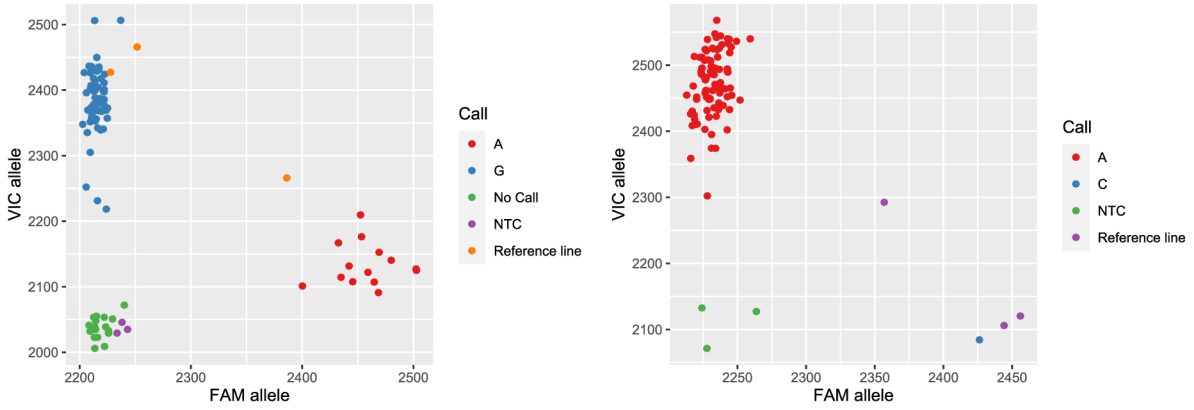

Figure 13: Allelic discrimination plots for KASP markers 6BL_IWB10724 (left) and 6BL_IWB72471 (right). The 96 samples consist of a panel of 90 winter wheats, three reference lines with a reported presence of Sr11 gene, and three wells with no DNA (NTC stands for no template control). Fluorescence is measured in relative fluorescence units (RFU).

7.3. Adult phenotyping methods and unknown resistance

This study presents a rare comparison of three disease phenotyping methods on both seedling and adult plants from a controlled experiment. Visual assessment is the most common method and provides fast results, even on a large dataset (Bock et al. 2022). While it does not require any expensive tools, qualified and experienced personnel is needed for accuracy and uniformity of results (Bock et al. 2015, 2016). Second method used here is image analysis based on machine learning. While also evaluating visual disease symptoms, this method provides a more objective quantification because the same classifier (a model) is used on the entire dataset and thus human error is eliminated. The classifier in this model also quantifies pustules and chlorosis separately as distinct symptoms. This is hardly possible with visual estimate. Third method used is quantification of fungal chitin inside the diseased samples (Ayliffe et al. 2013).

The WAC is especially valuable because it does not analyse the visual symptoms but instead quantifies the total mass of the pathogen that has accumulated in the host. While this method is relatively simple and provides relative quantification, it should be used with care because it is not specific to rust fungi. The WGA-FITC, a central component of the protocol, binds to chitin from any fungi and possibly even some leaf components, and even mock-inoculated samples record some fluorescence in the results. Here, the same samples were used for all the three methods so that they can be compared. Same leaves were scored visually, then scanned for image analysis, and then ground into powder for WAC. In Figure 14, visual assessment is compared to image analysis and WAC assay. The LOESS regression is used to plot the trendline and visualize the data distribution patterns. When reactions are moderately resistant to moderately susceptible, the visual scoring seems to have higher resolution. The actual area of disease symptoms quantified by image analysis seems to grow exponentially in the susceptible reactions. Only one sample was rated 9, which corresponded to more than one fifth of the leaf covered with symptoms. Literature suggests that 37% coverage of leaves with rust symptoms can be assumed to cause maximum damage to the host plant and higher intensities are rarely observed (Peterson et al. 1948).

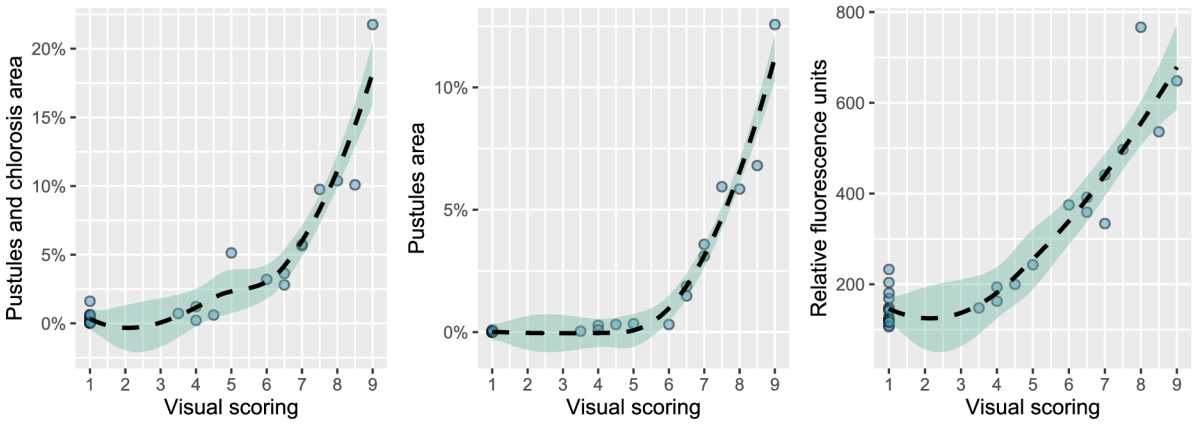

Figure 14: Visual scoring of disease severity plotted against image analysis results and measured values from chitin quantification done with adult plant leaves. Left: Visual scoring against leaf area covered by pustules and chlorosis. Middle: Visual scoring against leaf area covered by pustules. Right: Visual scoring against relative fluorescence units following WGA-FITC staining of chitin inside the leaves. Loess method was used to plot a trendline in all scatterplots to show patterns in the data distribution.

WAC assay results can be compared to disease symptoms quantified by image analysis (Figure 15). While the image analysis measures disease severity in terms of visible symptoms, the chitin quantification targets the amount of pathogen in the tissue. It appears that the results from the two methods correspond less when the disease severity is higher, although more data points would be needed to make a precise assessment. In theory, rust mycelium develops inside the leaf tissues before the pustules and/or chlorosis appears on the surface of the plant. Sporulation rates between individual genotypes of the pathogen have also been shown to differ. It is possible that quantifying chitin inside the leaves provides more information about the actual colonization of the plant compared to the visual assessments. It would be interesting to run both the WAC assay and a qPCR quantification on the same set of samples. However, qPCR quantification in wheat rust pathosystems is seldom used (Maree et al. 2020; Prabhakaran et al. 2021; Maré et al. 2021). In our lab, it was attempted unsuccessfully using samples discussed here (data not shown).

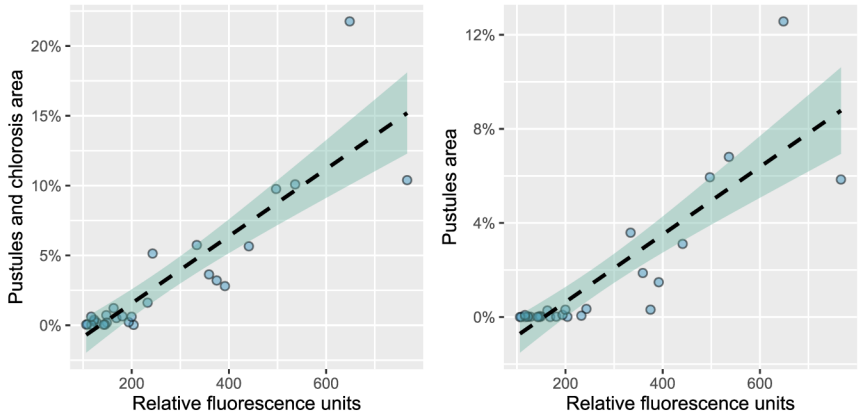

Figure 15. Scatterplots showing chitin quantification results (WAC) plotted against image analysis results. Data from adult plant leaves. Trendline plotted using linear model. Left: Leaf area covered by pustules and chlorosis combined; right: Leaf area covered by chlorosis.

In this experiment, seven lines were analysed for their seedling and adult response to two important races of *Pgt*. LG Mocca was shown to be the most susceptible cultivar both in seedling and adult stage and compares only to the susceptible control Michigan Amber. This is alarming as LG Mocca was the most widely propagated cultivar in Czech Republic in 2022 according to the reported areas (Central Institute For Supervising and Testing In Agriculture 2022). Results demonstrate the importance of adult plant rust phenotyping on case of Evina and Rivero (Figure 16). Seedlings of these two cultivars show moderate disease symptoms with more chlorosis, but adult plants show almost zero disease symptoms. Evina and Rivero should be studied further to describe the genetic basis of their adult plant resistance.

Rivero seedling infection was rated higher by visual scoring and lower by WGA and image analysis. This is probably because visual scoring takes the size of the pustules into account and may result in higher scores even when infection is not distributed uniformly. This shows that while image analysis evades human error in the measurement itself, uniform samples are crucial. Results of image analysis should always be compared to visual assessment to avoid misinterpretation of results. Since both pustules and chlorosis areas are quantified, their ratios can be calculated easily. Interestingly, this provides the only value which is fully independent on the uniformity of infection. Chlorosis is the result of an resistant reaction. The amount of pustule area was only higher than the chlorosis area in the most susceptible plants in the panel (ratio > 1.0). This measurement can be considered as one of the novel outputs of the image analysis, showing abundant sporulation combined with relatively weaker resistant reaction. However, this measurement should only be compared between images classified with the same model, as different models will have different thresholds for each class.

According to my knowledge, this is the first time that Trainable Weka Segmentation plug-in of ImageJ (Arganda-Carreras et al. 2017) was used for wheat rust phenotyping. Results reported here show that it is a fitting addition to the array of phenotyping methods. Its main advantages are the speed, almost no technical requirements, and no required programming skills. Pictures are best taken with a common document scanner (recommended resolution = 600 dpi). The software is free and utilizes machine learning, therefore each model can be adapted to the dataset in question. This method allows to bulk many repetitions into one scanned image (if it fits the scanner format), and the result is a simple proportion of disease symptom areas.

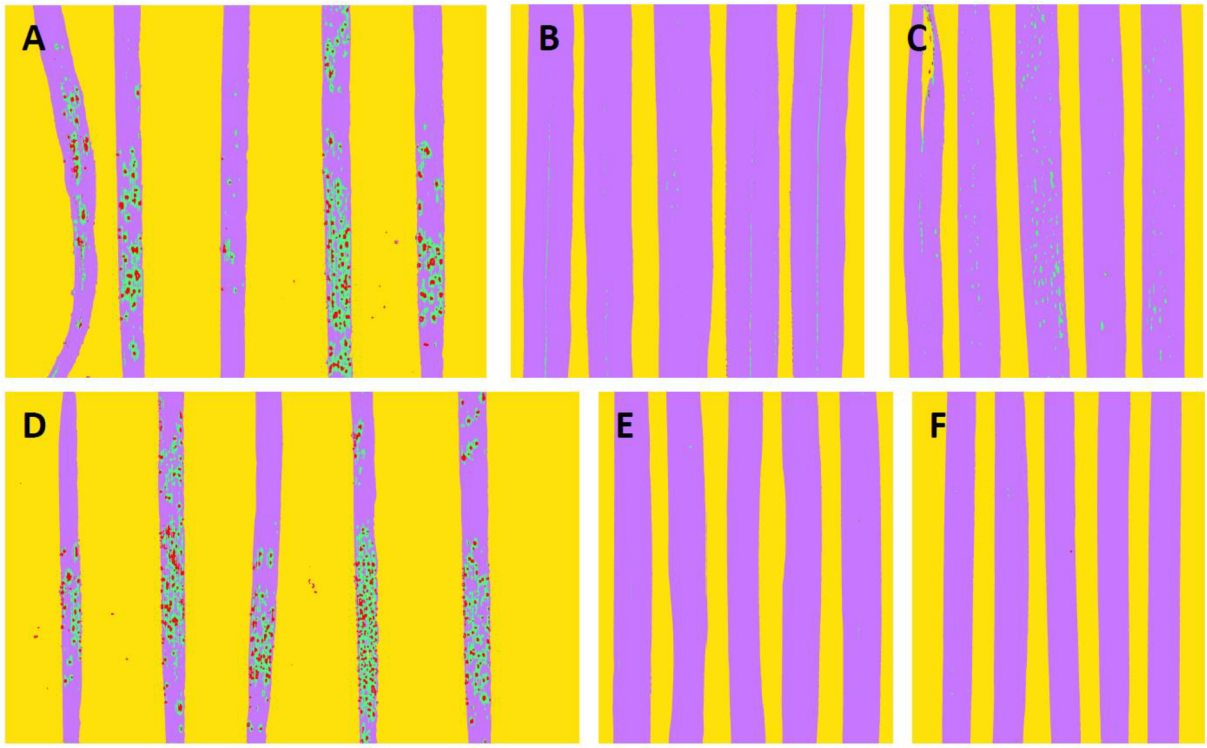

Figure 16: Image segmentation in infected leaves photographs of wheat cultivars Evina and Rivero. Red pixels indicate pustules, green pixels indicate chlorosis, purple pixels indicate healthy portion of leaf, yellow pixels indicate background. All samples in images were inoculated with TRTTF race of Puccinia graminis f. sp. tritici. A. Evina seedling; B. Evina flag leaf; C. Evina flag -1 leaf; D. Rivero seedling; E. Rivero flag leaf; F. Rivero flag -1 leaf.

8. Conclusions

In summary, presented work uses molecular methods and phenotyping to update the knowledge of genetic resistance of Czech cultivars of winter wheat to stem rust. It presents a continuation of studies of stem rust done locally but also puts this knowledge in context of global studies of this plant disease.

Field resistance trials in recent years showed a wide range of responses from completely resistant to completely susceptible, suggesting a variety of resistance genes in the genomes. With the use of several molecular markers, presence of several major genes for resistance can be assumed. More than half of the tested cultivars probably carry *Sr38*, and those have better results in the field tests. However, this gene is largely overcome by newly spreading races from the tropics and its effect is lost against those. In lower frequencies, *Sr24* and *Sr31* also appear, and susceptibility to those races has not been detected in Czech Republic yet. However, races of the pathogen that overcome resistance conferred by those genes have been described in other areas from which they may easily spread to Central Europe. In some cultivars, greenhouse and field trials showed resistance that could not be explained by the range of available molecular markers, and this could either be conferred by known or yet undescribed resistance genes. Several markers linked to other resistance genes (*Sr2*, *Sr15*, *Sr36*, and others) yielded no positive results in our selection, showing that those genes have either disappeared from local wheat, or were never introduced.

In this study, several KASP markers linked to *Sr8a* and *Sr11* were tested against local germplasm, a panel of 90 wheats. The marker assays yielded contradicting results and phenotypic tests will have to be made to determine their usefulness.

Some cultivars showing unusual stem rust reaction patterns were then picked for controlled adult plant phenotyping experiments using two previously described pathogen races. These tests confirmed that the most widely grown cultivar in the Czech Republic at this time is also completely susceptible to stem rust, even surpassing the usual susceptible checks. Furthermore, some lines do appear to possess adult plant resistance that would otherwise go unnoticed using other phenotyping methods, thus proving the validity of such laborious methods. These tests were supported with chitin quantification of samples and image analysis using machine learning. This image analysis method was used for the first time in stem rust pathosystem and is thus validated in this study as a useful tool to support visual estimations.

9. Future perspectives

The durability of resistance described in this study will be put to test during future growing seasons, as it will depend on the virulence of stem rust races being transferred from other areas. Future shifts in pathogen populations should be monitored further by sampling and analysis. It will be beneficial to include genotypic data in the analyses.

To shed more light on the resistance in local cultivars, more markers can be used in future, once they are available. There are ongoing attempts by laboratories to convert existing markers to KASP assays and publish new ones. To follow up on the *Sr8a* and *Sr11* SNP assays done here, phenotypic work with races polymorphic in virulence to these genes could be done, to conclude whether at least some of the markers give relevant results in local germplasm.

Our panels revealed cultivars with differential response to distinct races. Those must contain major genes and it would be helpful to add them to the differential set for race typing. Furthermore, a project focusing on genomic breeding for rust resistance is underway, led by the Crop Research Institute, which will shed light on the QTLs conferring horizontal resistance and will possibly explain some of the yet uncharacterised major gene resistance.

Adult plant experiments are very tedious and can only be continued with a small panel of wheats, such as in this study. This will only be useful with cultivars having contrasting seedling and field response. Image analysis method presented here can be further used to support visual phenotypic data, especially in cases where precise numbers are needed for statistical evaluation.

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11. Ph.D. candidate information

11.1. List of published articles

Zelba, O., Hanzalová, A., Dumalasová, V., & Viehmannová, I. (2022). Analyzing wheat cultivars grown in Czech Republic for eight stem rust resistance genes. European Journal of Plant Pathology, 162, 221–230. https://doi.org/10.1007/S10658-021-02397-3

Hanzalová, A., & **Zelba, O.** (2022). Leaf rust (*Puccinia triticina* Eriks) resistance genes in wheat cultivars registered in the Czech Republic. Journal of Plant Diseases and Protection, 1–7. https://doi.org/10.1007/S41348-022-00625-4

Hanzalová, A., Dumalasová, V., & **Zelba, O.** (2021). Virulence in the *Puccinia triticina* population in the Czech Republic and resistance genes in registered cultivars 1966–2019. Euphytica, 217(1). https://doi.org/10.1007/s10681-020-02733-4

Hanzalová, A., Dumalasová, V., & **Zelba, O.** (2020). Wheat leaf rust (*Puccinia triticina* Eriks.) virulence frequency and detection of resistance genes in wheat cultivars registered in the Czech Republic in 2016-2018. Czech Journal of Genetics and Plant Breeding, 56(3), 87–92. https://doi.org/10.17221/86/2019-CJGPB

11.2. List of conference contributions

Zelba, O., Hanzalová, A., Dumalasová, V. (2020). Wheat leaf rust (*Puccinia triticina* Eriks.) virulence frequency and detection of resistance genes in wheat cultivars registered in the Czech Republic in 2016-2018 (Poster). Borlaug Global Rust Initiative Technical Workshop 2020, 7-9 October, 2020. Virtual

Zelba, O., Hanzalová, A., Dumalasová, V. (2021). Poster: Wheat resistance to rust diseases in Czech Republic negatively correlates with baking quality and mostly relies on *Lr37/Yr17/Sr38* gene cluster (Poster). 16th ICC Cereal and Bread Congress hosted by International Association for Cereal Science and Technology, 29-31 March, 2021. Virtual

Zelba, O., Hanzalová, A. (2022). Poster: Physiologic specialization of wheat leaf rust (*Puccinia triticina* Eriks.) in the Czech Republic in 2016-2021 (Poster). International Cereal Rusts and Powdery Mildews Conference 2022, 31st August – 2nd September 2022, Cambridge, UK

Zelba, O., Hanzalová, A. (2022) Výskyt rzí v Evropě v posledních pěti letech (Wheat rusts in Europe during last five years) (Oral presentation). Pšenice 2022 hosted by Crop Research Institute, 1-2 December 2022, Prague, Czech Republic

11.3. Author contribution statement

I declare that I contributed to the listed publications with experimental design, laboratory work, data collection, and data analysis of the laboratory experiments. I also contributed to field experiments that were organized by Mgr. Alena Hanzalová, Ph.D., who also prepared the inoculum for the experiments. I wrote the manuscript of the first author publication, reviewed and approved the other manuscripts. All other work described in this thesis was conceived and conducted by me unless specifically stated.

11.4. Internship

Aarhus University, Department of Agroecology: Global Rust Reference Center. September-October 2021

11.5. Author's identifiers

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11.6. Funding

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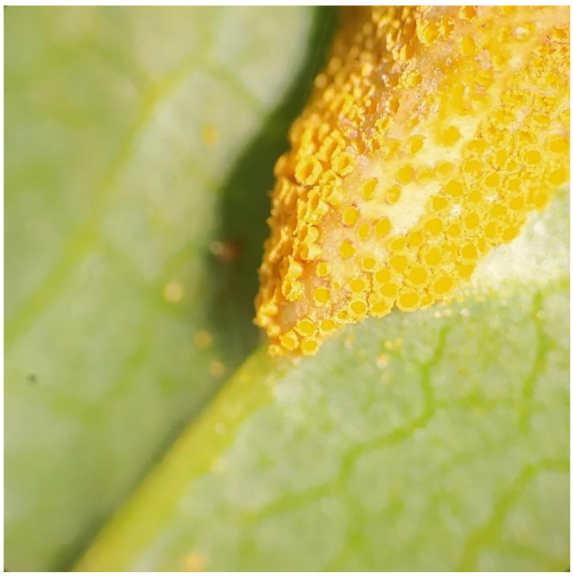
12. Appendix

12.1. Photos of *P. graminis* mycelia on barberry

Following photos show *P. graminis* on barberry (*B. vulgaris*) leaves collected in May and June 2022 in Czech Republic. Presumably, these belong to subsp. *graminicola*, as infection of wheat with aeciospores was not successful. Photos show pycnia on the top side of the leaves.


Aecia with opened aecial cups on the bottom side of barberry leaves.







P. graminis aecia colonized by a hyperparasitic fungus, possibly *Tuberculina persicina*.

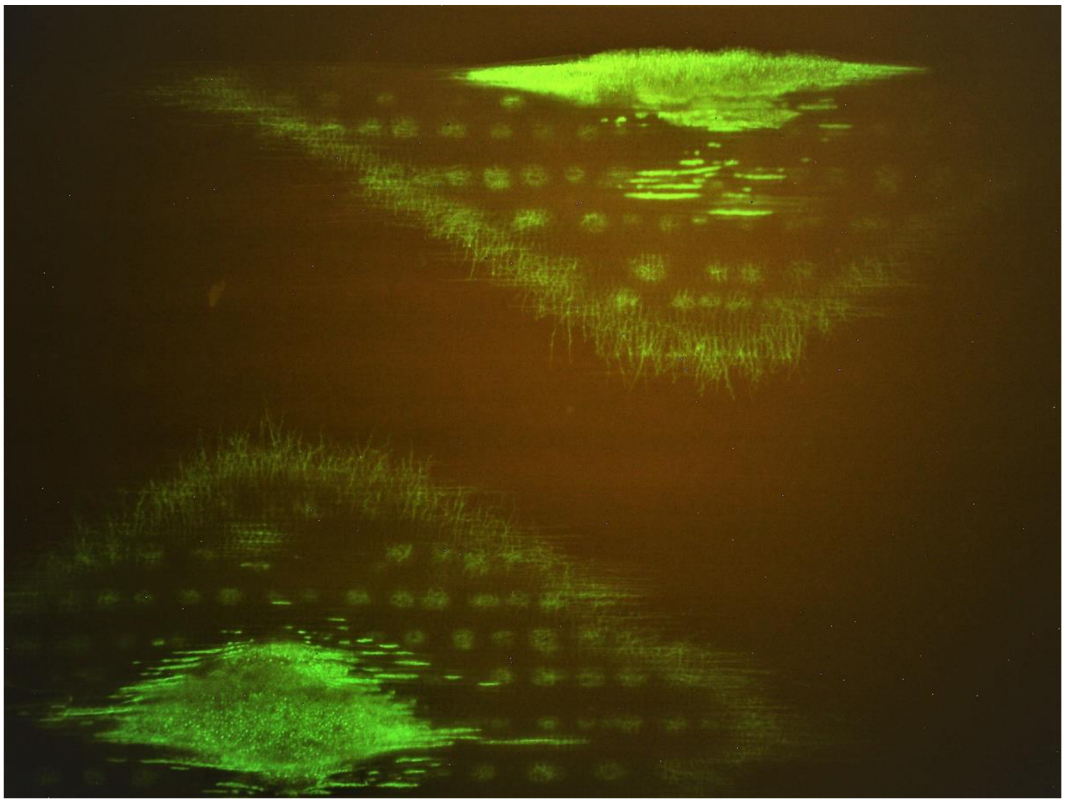
Aecia forming on barberry fruits.




Barberry leaves under heavy infection pressure, with many fungal colonies.

12.2. Miscellaneous photos

Stem rust disease on a susceptible wheat seedling ten days after inoculation using fluorescent microscopy. Two pustules are visible with the mycelium underneath. There is a healthy leaf area in between the two infection sites.



Stripe rust pustule with spores under fluorescent microscopy.