Genetic identification and characterisation of gain-of-virulence mutants of *Puccinia graminis* f. sp. *tritici*

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Abstract

The fungus *Puccinia graminis* f. sp. *tritici* (*Pgt*), is the causal agent of wheat stem rust, a disease that has regained attention after the emergence of virulent races which have caused epidemics in Africa, Europe, and Central Asia. Durable control of this disease requires the development of wheat varieties that contain several cloned stem rust resistance (*Sr*) genes that are stacked together at a single locus. However, some resistance genes interact negatively or fail to function when expressed in some genetic backgrounds. Thus, verification of the function of stacked *Sr* genes must be conducted after stacking. The use of the pathogen in these assays is difficult due to the simultaneous secretion of multiple avirulence (Avr) effectors. The heterologous expression of single *Avr* effectors can avoid this limitation. However, this strategy is hampered by the availability of only a very few (three) cloned *Pgt Avrs* to test *Sr* stacks. To accelerate *Pgt Avr* gene cloning, I outline a method to generate an ethyl methanesulphonate (EMS) mutant *Pgt* population followed by screening for gain-of-virulence mutant isolates. I treated urediniospores with EMS and created a library of > 12,000 mutant isolates. I selected random mutants for sequencing and established the average EMS transitions to be 1 single nucleotide variant (SNV) per 258 kb. I screened the mutant library on wheat seedlings carrying *Sr43*, *Sr44*, or *Sr45.* From this, I obtained 9, 4, and 14 *Pgt* mutants with virulence toward *Sr43*, *Sr44*, and *Sr45*, respectively. Upon isolation and reinoculation of the mutants onto the lines they were identified, only the mutant isolates on *Sr43* and *Sr45* showed stable virulence. I characterized 8 mutants virulent on *Sr43* by checking their virulence profile on the stem rust international differential set containing 20 defined *Sr* genes. These mutants maintained the same virulence profile as the wildtype from which they were derived showing that they were not contaminants. I further characterised two mutants, E1 and E7-1, to quantify their growth on *Sr43* via chitin fluorescence. There was no difference between the chitin fluorescence of these mutants on *Sr43* and that of the wildtype on the recurrent parent Chinese Spring. Therefore, loss of AvrSr43 has no apparent effect on *Pgt* fitness. In conclusion, my method enables the selection for virulent mutants toward targeted resistance (*R*) genes. The mutant library can be created from as little as 320 mg spores which provides a resource that enables screening against several *R* genes without repeating the EMS mutagenesis.

Table of contents

List of figures

List of tables

List of abbreviations

AGO	Argonaute protein family
APR	Adult Plant Resistance (resistance genes active only at plant maturity)
Avr /Avr	Avirulence gene/ protein
CIMMYT	Centro Internacional de Mejoramiento de Maíz y Trigo (International Maize and Wheat Improvement Center; Mexico)
ETI/S	effector-triggered immunity/ susceptibility
HR	hypersensitive response
Kbp	kilo base pair
Lr/ LR	Leaf rust resistance gene/ protein
Mbp	million base pair
MITE	Miniature inverted-repeat transposable elements
NCBI	The National Center for Biotechnology Information
NGS	Next-generation sequencing
NLR	Nucleotide-binding and Leucine-rich repeat immune Receptors
PAMP	Pathogen-Associated Molecular Patterns
Pc	*Puccinia coronata* (oat crown rust fungus)
PCR	Polymerase chain reaction
Pgt/ PGT	*Puccinia graminis* f. sp. *tritici* (stem rust fungus)
Ph	*Puccinia hordei*
PRR	Pattern recognition receptors
Pst	*Puccinia striiformis* f. sp. *tritici* (stripe rust fungus)
Pt	*Puccinia triticina* (leaf rust fungus)
PTI	PAMP-Triggered immunity
R gene	Resistance gene
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variation
Sr/ SR	Stem/ black rust resistance gene/ protein
Yr/ Yr	Yellow/ stripe rust resistance gene/ protein

Lr/ Lr Leaf/ brown rust resistance gene/ protein

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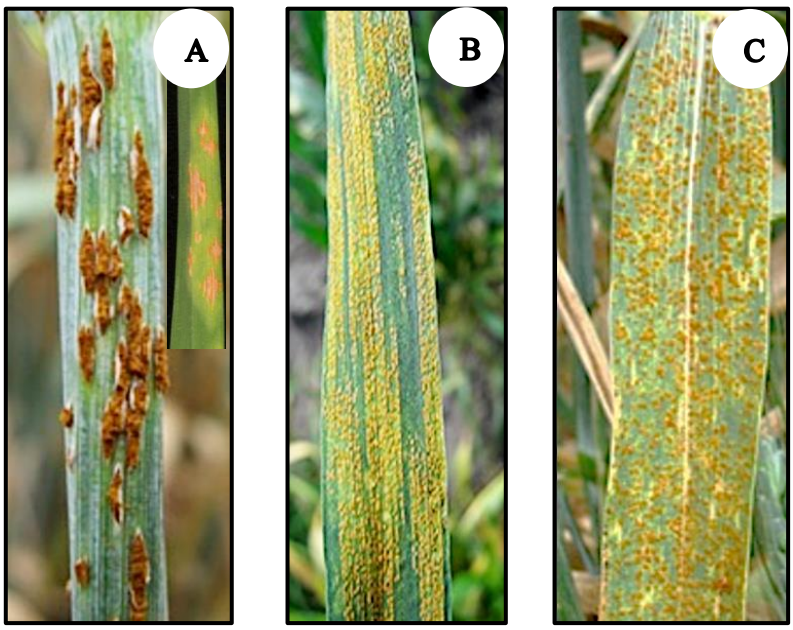
1. Chapter 1. Literature review.

1.1. Wheat stem rust is a threat to global wheat production.

Wheat (*Triticum aestivum* and *T. turgidum* var. *durum*) is cultivated on more land area than any other cereal globally with more than 215 million hectares worldwide and an estimated annual production of over 766 million metric tons valued at over US$ 150 billion (FAO 2019). Bread wheat (*T. aestivum*) constitutes 95% of this production (Tadesse et al. 2019). Wheat is a source of approximately 25% of protein and 20% of calorie intake globally and this can be as high as 50% among the 1.2 billion people dependant on wheat consumption in developing countries (Oerke 2006; Pingali 2007). With the world population expected to double by 2050, agricultural production is projected to need to increase by between 60% and 110% to meet the demand for food (Godfray et al. 2010). However, the annual increase in wheat yield as a result of genetic gain from wheat breeding programmes is currently at 1% (Tadesse et al. 2019) versus a required rate of 2.4% to meet future demands (Ray et al. 2013).

These genetic gains are constantly threatened by pests, diseases, and weeds as well as abiotic factors like climate variability, soil fertility and salinity. Wheat diseases alone account for an estimated annual grain yield loss of 21% (Savary et al. 2019a) In particular, the three wheat rust pathogens: *Puccinia graminis* f. sp. *tritici* (*Pgt*), the causative agent of stem (or black) rust disease; *Puccinia striiformis* f. sp. *tritici* (*Pst*), the causative pathogen of stripe (or yellow) rust disease and *Puccinia triticina* (*Pt*), the causative agent of leaf (or brown) rust disease, are important (Fig 1.1). Annual losses attributed to these three diseases is around US$ 5.0 billion (Figueroa et al. 2018). Of this amount, US$1.2 billion –equivalent to 6 million tonnes, or 8.4 million English loaves– is predicted to be due to the impact of wheat stem rust alone (Pardey et al. 2013). These global figures of losses are not representative of what occurs at local levels where entire crops can be destroyed (Schumann and Leonard 2000; Dean et al. 2012). This is especially so in developing countries where warm weather and low use of fungicides due to costs favour disease development. Therefore, reducing wheat yield losses due to wheat stem rust as well as other diseases by breeding for resistance can contribute

significantly to ensuring increased food availability complementing the efforts to improve the current genetic yield potential. Most of the major wheat production areas worldwide provide favourable environments to support the growth of *Pgt*. It is estimated that 90% of wheat grown in the world is susceptible to stem rust (Ravi P. Singh et al. 2015).



Stem rust Stripe rust Leaf rust

(Roman-Reyna 2017)

Figure 1.1 | Infected wheat showing pustules (uredinia) that are reddish brown in *Puccinia graminis f. sp. tritici* on the stem and leaf (inset, (A)), yellow in *Puccinia striifromis* f. sp. *tritici* (B), and brown in *Puccinia triticina* infections (C).

1.1.1. The identification of isolate *Pgt* UK-01 (race TKTTF) in the United Kingdom.

In 2013, wheat stem rust was identified in the United Kingdom on a single plant in a breeding plot in Suffolk county after close to 60 years of absence within the country as the last recorded epidemic was in 1955 (Lewis et al. 2018). As mentioned earlier, This was the same year when outbreaks occurred in Germany, Sweden, Denmark and Ethiopia (Olivera *et al.*, 2015). The isolate identified in the UK was called *Pgt* UK-01 and differential analysis showed the race type to be TKTTF. Section 1.3 contains a description of how *Pgt* races are determined based on their virulence/ avirulence on an international standard stem rust differential set comprising 20 lines, each carrying a specific stem rust resistance gene. This race typing along

with phylogenetic analysis showed *Pgt* UK-01 to be identical to isolates identified in other parts of Europe and East Africa but it is not clear how this and 80% of the commercial wheat varieties in the UK are vulnerable to it (Lewis et al. 2018; Kangara et al. 2020). In addition, it was discovered that there were barberry plants that were infected by aecia from *Pgt*, *P. graminis* f. sp. *secalis* from wild rye and *P. graminis* from couch grass. These, however, do have the ability to infect wheat to different extents (Lewis et al. 2018). Following this, stem rust was identified on barley late in the season from June to August. The plants, which had both uredia and telia, were located close to *B. vulgaris* which had aecia. ITS genotyping of aecia samples from infected barberry showed the same mix of *P. graminis* formae speciales as identified in the analysis of samples collected in 2017 which were indicative of both uredia and telia on the barley (Orton et al. 2019).

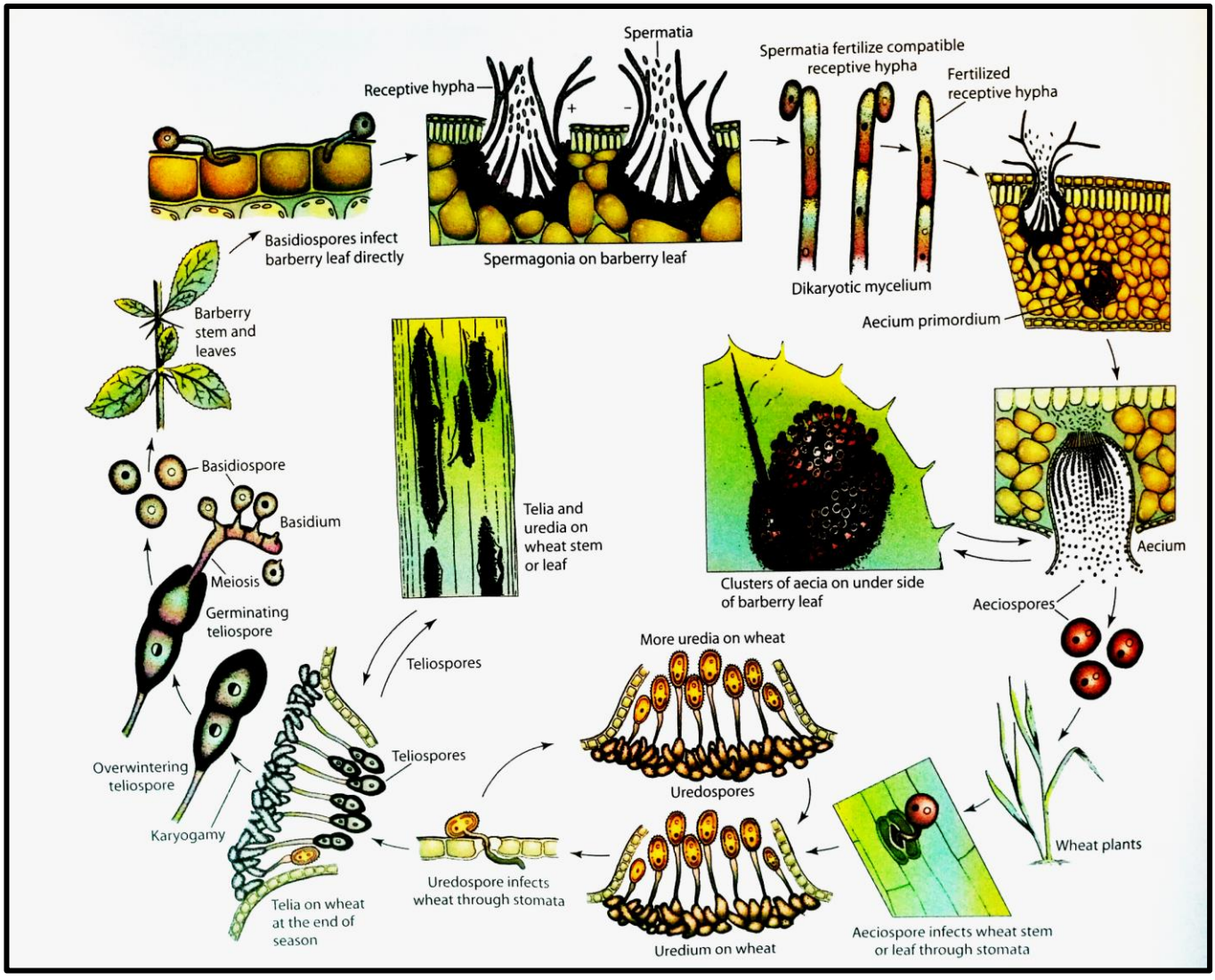
In a bid to save the decreasing population of the barberry carpet moth, *Pareulype berberata*, conservationists have started the replanting of barberry and this poses a threat to barley and wheat production (Orton et al. 2019; Lewis et al. 2018). It also highlights how stem rust control needs a collaborative effort between different interest groups. These could result in solutions like the re-planting of barberry at distances where the windborne aeciospores do not reach crop fields. With a single bush producing over 6 billion aeciospores, this might be as serious challenge (Schumann and Leonard 2000) depending on how mobile the aeciospores are. Another solution is searching for alternative food sources for the barberry carpet moth and eradicating barberry bushes altogether or finding a barberry species that is palatable to the moth but is resistant to *Pgt* infection. These are being investigated by the John Innes Centre in partnership with other stakeholders (Diane Saunders, personal communication).

The identification of *Pgt* UK-01 in the United Kingdom presented an opportunity for the work in this PhD to utilise an isolate belonging to the important “Digalu race” that is prevalent in Africa, the Middle East and Europe. In addition, the rarity of stem rust in the UK also presented a low risk to contamination from other *Pgt* isolates that could have different virulence profiles thereby confounding results in experiments involving screening mutagenized *Pgt* UK-01 spores on resistant lines to identify individuals with changes in virulence due to the induced

mutations. Finally, the biosafety protocols for handling *Pgt* UK-01 would be easier to implement compared to working with exotic *Pgt* isolates.

1.2. Biology of *Puccinia graminis* f. sp. *tritici*

1.2.1. Life cycle of *Puccinia graminis* f. sp. *tritici*



Reproduced from Agrios (2005)

Figure 1.2 | Life cycle of *Puccinia graminis* f. sp. *tritici.*

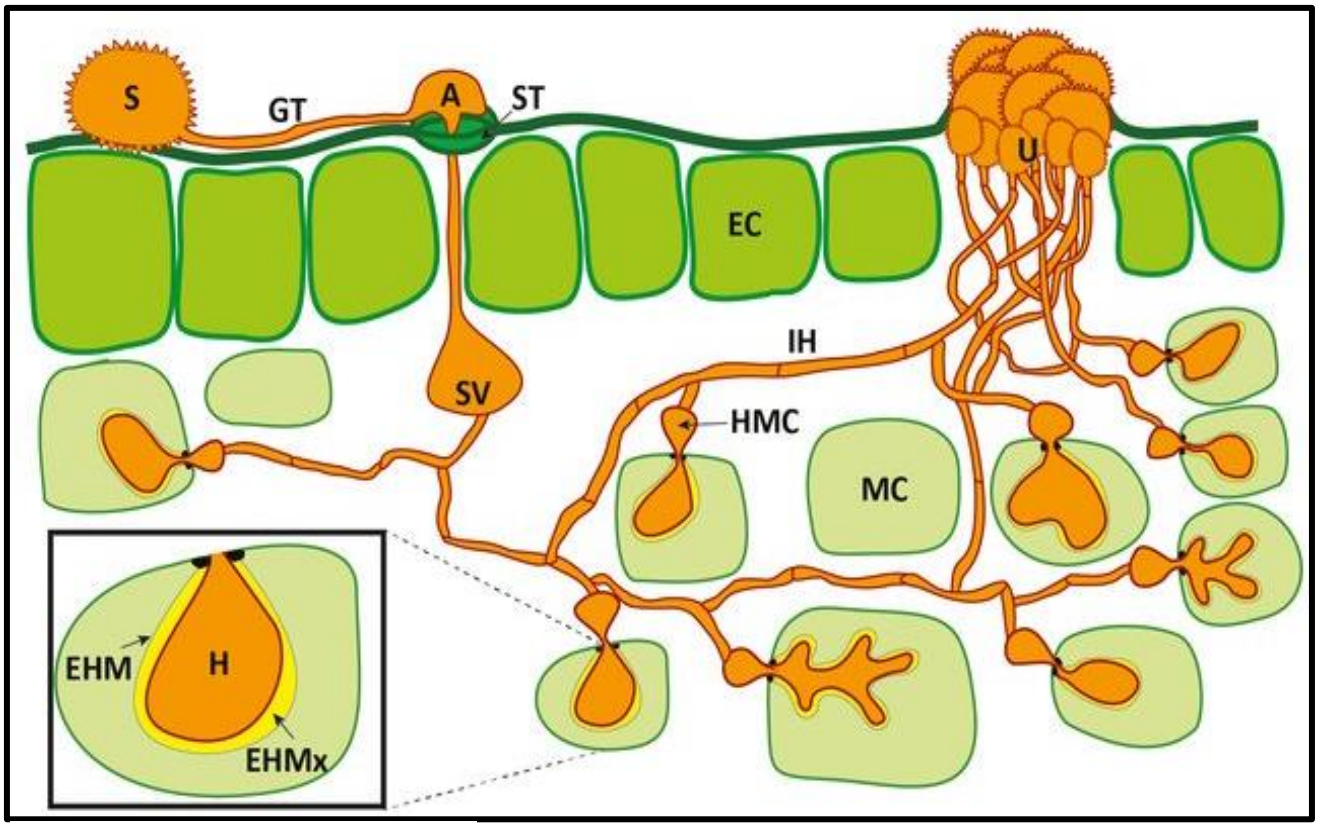
Pgt is a biotrophic, macrocyclic and heteroecious rust fungus (Schumann and Leonard 2000; Quilliam and Shattock 2003). It has a complex life cycle that comprises five different spore types namely, urediniospores, teliospores, basidiospores, pycniospores and aeciospores

(Schumann and Leonard 2000). Some of these stages are completed on compatible host plants belonging to the Gramineae family such as wheat, barley and Triticale on which asexual reproduction occurs and then others on the unrelated dicot species of the Berberidaceae family, *Berberis* and *Mahonia* on which sexual reproduction occurs (Agrios 2005). *Pgt* is a dikaryotic organism (n + n) meaning its genome is contained in two heterozygous haploid nuclei in each cell. Each nucleus has the full set of 18 chromosomes (Chen et al. 2017; Li et al. 2019). *Pgt* is complex in that it requires a living host to complete all these stages in its lifecycle and thus cannot be maintained under laboratory conditions on artificial growth media like many other fungi such as Botrytis, Magnaporthe or Fusarium. Thus, *Pgt* is grown on wheat using urediniospores or aeciospores. This requires larger facilities with enough space to keep pots containing inoculated plants during screening experiments. The facilities should have controlled conditions that can be set to the optimum day/ night temperature, light and humidity levels for the growth of the host and pathogen.

The asexual stage in the *Pgt* life cycle starts with the infection of the cereal host by the dikaryotic (n + n) aeciospores formed from sexual reproduction of *Pgt* on barberry and mahonia. Following successful infection, erumpent uredinia (pustules produce large quantities of dikaryotic urediniospores (n + n) which are dispersed from the leaf surface to cause new infections (Schumann and Leonard 2000). These infections on local or distant areas on new plants occur under favourable conditions such as temperature above 2 ^{o}C and dew formation on the surface of leaves where the urediniospores will have been deposited thereby producing new pustules (Singh, 2002). This cycle can be completed in as little as 8 days thus large amounts of inoculum can be produced in a summer season resulting in large areas of infection where susceptible hosts are grown. The asexual stage is advantageous for use in research as it easily infects wheat and does not require special conditions during the infection process. Genetic variability is unlikely during several rounds of infecting susceptible cultivars to bulk up inoculum. This allows maintenance of isolate and race purity for use in testing wheat cultivars. In addition, historical collections can be kept as spores remain viable for long periods after drying and storage under -80 ^{o}C or liquid nitrogen. This is the spore stage that was exploited in this thesis to maintain and bulk up *Pgt* UK-01 and it EMS mutant isolate derivatives.

Towards the end of the season, the fungus produces black teliospores on leaves and stems–thus the disease is also known as black rust–which are thick-walled overwintering structures. The phase of sexual reproduction commences when the teliospores which are initially dikaryotic become diploid as a result of fusion of the two heterozygous nuclei (karyogamy) resulting in a single diploid nucleus (Agrios 2005). When winter ends, environmental cues such as increasing temperatures and alternate wetting and drying initiate the diploid teliospores to germinate producing basidia that have four haploid basidiospores with single haploid nuclei (n) by meiosis. These basidiospores are windborne and are transferred to barberry bushes, the alternate host. Pycnia form on the adaxial surfaces of a barberry/ mahonia leaves which make pycniospores (n) and receptive hyphae (Berlin et al. 2017). Pycniospores from different pycnia fuse with receptive hyphae from a compatible mating type (Bushnell 1984). This results in the production of aeciospores which are dikaryotic (n + n) on the abaxial surface of barberry leaves which are then transferred to barley/ wheat thus completing the cycle. In the absence of the alternate host, *Pgt* can continue to spread asexually during the cropping season and volunteer plants and windborne urediniospores from areas with warm weather can introduce inoculum (Schumann and Leonard 2000).

1.2.2. The infection cycle of *Puccinia graminis* f. sp. *tritici* on susceptible cereal hosts



Reproduced from Garnica et al. (2014)

Figure 1.3 | **Infection cycle of the asexual cycle of rusts on wheat.**

In the presence of free water from dew, high humidity, rain or irrigation splash, a urediniospore (S) on the leaf surface germinates producing a germination tube (GT) (Garnica et al. 2014). The GT grows on the surface of the leaf and is guided by topographical signals (thigmotropism). Upon reaching the stomatal aperture, the GT forms an appressorium above the stomatal aperture (A) and enters the leaf interior through the stoma (ST), where it differentiates into a substomatal vesicle (SV) (Torabi and Manners 1989). Infection hyphae (IH) grow in the apoplast and the tips differentiate into haustorial mother cells (HMC) upon contact with the mesophyll cells (MC). The into haustorial mother cells enter the mesophyll cells and form haustoria (H). The haustorial membrane is enclosed by a fungal cell wall and the plant cell-derived membrane which form the extrahaustorial matrix (EHMx) (Quilliam and Shattock 2003). The EHMx forms an interface for interaction between the fungus and the host through which uptake of nutrients (sugar and amino acids) from the host or secretion of effectors into the plant cell cytoplasm occurs (W. Bushnell 1984; Mendgen et al. 2000). Upon formation of the initial haustorium, secondary hyphae develop, which colonize the apoplast, and continue the formation of more haustoria upon contact with other plant cells. Within 8 days, the invasive hyphae form spore-producing basal cells in the uredia (U) and new urediniospores break through the leaf epidermis thus completing the infection cycle (Mendgen et al. 2000). Abiotic factors such as light, CO_2 concentration and water deficit can impact the infection development (Yirgou and Caldwell 1963). It was believed that appressorium formation was light-dependent due to its effect on stomatal opening (Bushnell, 1984; Singh, 2002). However, recent use of improved microscopy technology which is less disruptive and more sensitive have shown that *Pgt* does not require light for penetration whilst in the apoplast, hyphal growth, and haustoria development appear to be dependent on light (Solanki et al. 2019).

1.3. Breeding for stem rust disease resistance.

Understanding of the life cycle of *Pgt* led to the realisation of the benefits of the removal of the alternate host barberry. This strategy reduced the number of *Pgt* variants since it was launched in the 1800s in Europe and later in the United States (Barnes et al., 2020). As many as 64 billion aeciospores are produced by *Pgt* on a barberry bush and they provide ample inoculum for the infection of the next crop of wheat (Schumann and Leonard 2000). However, outbreaks of *Pgt* still occur as a result of wind dispersed spores from areas where stem rust is prevalent (Agrios 2005). Genetic control combined with barberry eradication is a highly effective and inexpensive strategy that can be adopted in developing countries. In addition, it is more environmentally sustainable than the use of fungicides globally (Bockus et al. 2001). Varieties can carry different types and levels of rust resistance.

Domestication of wheat and selection by early farmers of cultivars with agronomic advantages resulted in the 'domestication syndrome' where traits were fixed and some important alleles from the wild parents of wheat were lost (Haudry et al. 2007). For example, the nucleotide diversity of domesticated *Triticum dicoccum* is 70% less than the wild dicoccoides, for durum wheat, the difference is 84% less than the wild relative and for *T. aestivum*, nucleotide diversity is 69% lower than that of wild *T. dicoccoides* (Haudry et al. 2007). In 1905, Rowland Biffen became the first to demonstrate the Mendelian inheritance of disease resistance working on *Pst* resistance in wheat presenting an opportunity to introduce variation to wheat and other crops to combat crop diseases (Bushnell 1984). This was further enhanced by the discovery that host specialisation of stem rust isolates depended on the kind of resistance genes in cultivars (Stakman and Levine 1922), and the gene-for-gene hypothesis based on the study of *Avr-R* gene pairs in flax rust which improved knowledge in plant-pathogen interaction (Flor 1955). Studies of potato blight and its interaction with its hosts of varying resistance led to resistance being initially classified in plant pathology as either vertical (complete) or horizontal (partial) resistance (Van der Plank 1963, 1968).

In breeding for rust control, resistance genes of both *R* and adult plant resistance (APR) classes are designated *Lr*, *Sr*, or *Yr* depending on whether they control leaf (brown), stem (black), or

yellow (stripe) rust, respectively, followed by a number unique to the specific gene. *R* genes confer pathogen race- or strain-specific resistance (also called "major gene resistance", or "gene-for-gene resistance"). This resistance functions from seedling stage to maturity (Ellis et al. 2014) and the phenotype conferred is strong and easy to identify in seedling nurseries or glasshouses due to the characteristic necrotic lesions on the leaves (termed the hypersensitive response) thus leading to their widescale use in breeding programmes (Ellis et al. 2014). This type of resistance is dependent on the recognition of the pathogen as a result of the interaction between the *R* gene in the host and its corresponding avirulence effector gene (*Avr*) in the pathogen (Dodds and Rathjen 2010). Despite the usually strong resistance by *R* genes, pathogens can rapidly evolve to overcome single resistance genes. Some *Sr* genes in Australia were discarded from wheat breeding programs, either because races that could overcome their resistance already existed or new virulent races of the fungus quickly appeared (Luig 1978). Some genes for major resistance to stem rust in wheat remained highly effective for many years. The most successful of these was *Sr31*, a gene from rye that was transferred into wheat by a complicated process of interspecific hybridization (Ellis et al. 2014; Pretorius et al. 2000). The effectiveness of *Sr31* caused wheat stem rust to decline to insignificant levels nearly everywhere in the world by the mid-1990s (Ra 1983). As mentioned earlier in this review, resistance conferred by *Sr31* and other *R* genes like *SrTmp* and *Sr24* was eventually overcome by Ug99 which has since expanded its virulence to other resistance genes as different isolates developed mutations within the corresponding effector to each gene through spontaneous mutations, sexual reproduction and/ or hybridisation (Singh *et al.*, 2011; Figueroa et al., 2020). The probability of the emergence of virulent *Pgt* races increases if race-specific resistance genes are the only source of resistance in cultivars released to farmers leading to what is called the "boom-and-bust" cycle (Eversmeyer and Kramer 2000). Resistance is breakdown typically within 5 years or less of being deployed due to shifts in pathogen populations (Eversmeyer and Kramer 2000).

The second class are the APR genes that function mainly at the adult stage. Race-nonspecific, or "slow rusting" resistance (Van der Plank 1963) was initially applied to leaf rust resistance breeding programmes by Centro Internacional de Mejoramiento de Maíz y Trigo/ (International Maize and Wheat Improvement Center; Mexico (CIMMYT) utilising four to five

genes, each with minor effect but providing near immunity within a single cultivar (Khan et al. 2013). Genes conferring slow rusting resistance to leaf rust in wheat have partial and additive effects, and although the response to infection is essentially susceptible, the rate of disease progress is decreased. Some APR genes provide resistance to all isolates of a rust pathogen species and a subclass of these provides resistance to several fungal pathogen species. For example *Lr34*, confers partial resistance to stem rust, leaf rust, powdery mildew, stripe rust and wheat blast (Krattinger et al. 2016; Lagudah et al. 2009). APR genes typically confer partial resistance which results in constrained pathogen development and are thus at times referred to as slow rusting genes (Ellis et al. 2014). *Lr34* was cloned and can be used in gene stacks in combination with *R* genes with different effector specificities in order to provide durable resistance (Krattinger et al. 2009). Currently breeders and plant pathologists agree that increased emphasis should be placed on the identification and use of APR genes for pyramiding or stacking with *R* genes for durable resistance.

During the green revolution, semi-dwarf wheat varieties developed by Norman Borlaug under sponsorship by the Mexican Government and the Rockefeller Foundation were early maturing and resistant to stem rust (Singh et al. 2008). Genes like *Sr2* and alien resistance genes *Sr24* and *Sr26* from *Thinopyrum ponticum*, *Sr31* from Rye, *Sr36* from *T. timopheevi*, *Sr38* from *T. ventricosum* controlled stem rust disease in the 1970s and 1980s (Singh et al. 2008). *Sr31* was the most widely used gene in the 1980s because the translocation carrying it was associated with increase in grain yield and resistance to stripe rust, leaf rust and powdery mildew (Singh et al. 2008). Over time, around 60 genes for race specific resistance have been identified in wheat or transferred to wheat by wide crosses with wild relatives of wheat (Olivera et al., 2018). Of these, it seems most resistance genes sourced from wheat wild relatives appeared to have better control against Ug99 and race group than *Sr* genes of wheat origin (Jin et al. 2007; Singh et al. 2006). Amongst the wild relatives of wheat, *Aegilops* is the most closely related genus to *Triticum* (Jiang et al., 1993). It forms a large genetically diverse pool that is made up of 22 species and five untypical varieties with diploid and polyploid (tetraploid, and hexaploid) genomes (Van Slageren 1994). As such, several *R* genes have been transferred from *Aegilops* into cultivated wheat such as *Sr33*, *Sr39*, *Sr45*, *Sr53*, *Sr1644-1Sh*, *Sr-1644-5Sh*, *SrTA10187* and *Sr2020* (Mago et al. 2009; Sambasivam et al. 2008; Liu et al. 2011; Wiersma et al. 2016; Yu et al. 2017). Of these, *Sr33*, *Sr45*, *Sr1644-1Sh* and *SrTA1662* have been cloned

(Periyannan et al. 2013b; Steuernagel et al. 2016; Arora et al. 2019). The APR genes *Lr34* and *Sr2* provide partial resistance and have been used as in combination with other *R* genes to provide adequate rust resistance (Gepts et al. 2016). The *R* gene *Sr12* does not provide strong resistance to stem rust on its own but was shown to have increased resistance in combination with *Lr34* (Hiebert et al. 2016).

The application of wide crosses to re-introduce some of the rich diversity of resistance genes from wheat wild relatives though promising is not straightforward. The commercial incentive to utilise wild relatives in resistance breeding is also low. It takes ~6 years to combine traits from two wheat cultivars and to conduct trials where progeny that have a yield advantage that is at least 3% higher than the most productive parent is selected (Brown and Rant 2013). It takes much longer to do this when introducing traits from wild relatives into commercial cultivars and thus the yield advantage conferred by the introgression must be higher. The companies thus shun this lengthy and costly endeavour and settle for lower gains that results from crossing elite cultivars (Brown and Rant 2013).

In addition, using wild species for the transfer of beneficial genes into crops requires several cycles (years) of backcrossing to remove detrimental genes (linkage drag). In the cases of *Sr33*, *Sr45*, and *Sr46* from the wild diploid wheat *Aegilops tauschii* these have been associated with male sterility, poor endosperm quality and yield reduction (Ford-Lloyd et al. 2011; Rouse et al. 2011; Niranjana 2017). The *Sr11* gene is linked to the *ki* gene which results in unviable pollen thus impacting on how the R gene is inherited during crosses (Loegering and Sears 1963). The gene *Sr43* from *Th*. *ponticum* (also the source of *Sr24* and *Sr25*) is associated with high grain yield in addition to rust resistance, however, it is tightly linked with genes that give a yellow pigment to the flour produced from the grain (Niu et al. 2014). This linkage drag was broken through crossing wheat carrying *Sr43* with a Chinese Spring *ph1b* mutant and marker assisted selection for lines with shortened alien chromatin carrying the gene (Niu et al. 2014). In barley, *mlo* confers resistance to mildew, a biotrophic pathogen but increases susceptibility to necrotrophic and hemibiotrophic diseases (Brown and Rant 2013).

It should be noted that conventional wheat breeding programmes by companies and public institutions such as CIMMYT are now taking advantage of powerful technologies such as the application of genome wide markers to predict traits, development of double haploids,

genome editing (CRISPR-ready Cas9 seedlings), accurate automated large-scale phenotyping and speed breeding are accelerating conventional breeding (Meuwissen et al. 2001; Watson et al. 2018; Crossa et al. 2014; Hickey et al. 2019). These, however, are still expensive to implement.

1.4. Determining *Pgt* race types

Determination of *Pgt* virulence phenotypes is a key part of breeding programmes to develop resistant cultivars, as well as studying the geographic distribution and the evolution of virulence in *Pgt* populations. During his time as a postgraduate student, Elvin C. Stakman, under supervision by Edward Freeman, sampled rust isolates and cultivars across the United States. He found that there were 12 distinct races of *Pgt* within the US at that time (Stakman and Levine 1922). More importantly, this work showed that *P. graminis* had several stable forms. Each "physiologic race" was a unique set of individual isolates making up a *forma specialis* of *P. graminis*, as determined by the infection types they produced on hosts carrying defined resistance genes (Stakman and Levine 1922). As a result, physiological characteristics became important for classification due to differences in host resistance and not fungal morphology as previously determined by mycologists in the 16th to the 18th century (W. Bushnell 1984). This knowledge impacted breeding programmes significantly as predictable results could be obtained in tests of cultivars using specific pathogen races (Stakman and Levine 1922). The physiological race key developed by Stakman and Levine (1922) using 12 host lines each carrying a unique resistance gene, is still used widely and was updated to 16 host lines in 1962 (Stakman et al. 1962). More recently, it was updated to twenty host lines with the addition of *Sr24*, *Sr31*, *Sr38*, and *SrMcN*, to allow for distinguishing of isolates within the "Ug99 race group" that varied in their virulence/ avirulence against some genes that were not within the previous differential set (Table 1.1) (Jin et al., 2008).

Table 1.1 | International Core Stem Rust Differential Set.

Set	Gene	Differential line	Origin/Pedigree	Source
1	*Sr5*	ISr5-Ra CI 14159	Thatcher/Chinese Spring	Jin, USDA
	Sr21	T monococcum/8*LMPG-6 DK13	Einkorn CI 2433	Fetch, AAFC
	Sr9e	Vernstein PI 442914	Little Club //3* Gabo /2* Charter /3/3* Steinwedel / CI 7778	Jin, USDA
	Sr7b	ISr7b-Ra CI 14165	Hope/Chinese Spring	Jin, USDA
2	*Sr11*	Yalta PI 155433	Kenya C6402/Pusa4//Dundee	Park, Australia
	Sr6	ISr6-Ra CI 14163	Red Egyptian/Chinese Spring	Jin, USDA
	Sr8a	Mentana W1124 PI 221154	Rieti / Wilhelmina // Akagomughi	Park, Australia
	Sr9g	Acme CI 5284	Selection from Kubanka (CI 1516)	Pretorius, SA
3	*Sr36*	W2691SrTt-1 CI 17385	CI 12632 T. timopheevii	Jin, USDA
	Sr9b	Prelude*4/2/Marquis*6/Kenya 117A	Kenya 117A	Fetch, AAFC
	Sr30	Festiguay W2706 PI 330957	Festival / Uruguay C10837	Park, Australia
	Sr17	Prelude/8*Marquis*2/2/Esp 518/9	Esp 518/9	Fetch, AAFC
4	*Sr9a*	ISr9a-Ra CI 14169	Red Egyptian/Chinese Spring	Jin, USDA
	Sr9d	ISr9d-Ra CI 14177	Hope/Chinese Spring	Jin, USDA
	Sr10	W2691Sr10 CI 17388	Marquis*4/Egypt NA95/2/2*W2691	Jin, USDA
	SrTmp	CnsSrTmp	Triumph 64 (CI 13679)/Chinese Spring	Jin, USDA
5	*Sr24*	LcSr24Ag	Little Club/Agent (CI 13523)	Jin, USDA
	Sr31	Kavkaz/Federation4	Kavkaz	Pretorius, SA
	Sr38	VPM1	VPM	Park, Australia
	SrMcN	McNair 701 (CI 15288)		Jin, USDA

An isolate whose race is to be determined is first purified via single pustule isolation before inoculation onto the lines in the differential set (Woldeab et al. 2017). At 14 days after inoculation, the differentials are evaluated using the 0 – 4 scoring scale developed by Stackman et al. (1962), based on the classification of pustule size by visual inspection. The scores from 0 to 2+ scores are considered avirulent (resistant reactions) and 3 to 4 scores are considered virulent (susceptible reactions). A matrix with letter codes is used to determine the five letters denoting the race – one letter per set – based on the isolate's combination of virulence (H) or avirulence (L) per line within each set (Table 1.2). For example, the first isolate identified within the "Ug99 race group" is officially named TTKSK because of its virulence against all the lines in set 1 (T) and set 2 (T). In set 3 (K), it is avirulent on *Sr36* and virulent on S*r9b*, *Sr30* and *Sr17.* For set 4 (S), it is virulent on *Sr9a*, *Sr9d*, *Sr10* and avirulent on *SrTmp.* In the final set (K), *Sr24*, *Sr31*, *Sr38*, *SrMcN,* the isolate is virulent against all lines except *Sr24*.

The isolate used in this PhD research is *Pgt* UK-01. This isolate is virulent against all *Sr* genes in sets 1, 3, and 4. In set 2, the isolate is avirulent on *Sr11* but virulent on *Sr6*, *Sr8* and *Sr9g*. In set 5 *Pgt* UK-01 is avirulent against *Sr24* and *Sr31*, two genes that were widely deployed in cultivated wheat. Thus, *Pgt* UK-01 is designated TKTTF ("Digalu group"). The avirulence of *Pgt* UK-01 on *Sr31* is one of the major distinguishing factors between TKTTF and TTKSK, the two most widely distributed *Pgt* races.

Table 1.2 | North American Stem Rust Race Nomenclature Code Sheet.

	Four gene differential sets				
	Sr5	*Sr21*	*Sr9e*	*Sr7b*	Set 1
	Sr11	*Sr6*	*Sr8a*	*Sr9g*	Set 2
	Sr36	*Sr9b*	*Sr30*	*Sr17*	Set 3
	Sr9a	*Sr9d*	*Sr10*	*SrTmp*	Set 4
Pgt letter	*Sr24*	*Sr31*	*Sr38*	*SrMcN*	Set 5
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	

H = High Infection Type (3-4 on standard evaluation scale)
L = Low Infection Type (0-2 on standard evaluation scale)

1.5. The resurgence of stem rust.

1.5.1. The Ug99 and Digalu groups of races.

The success of stem rust control began through breeding to transfer resistance receptors (Sr) that between cultivars and from wheat wild relatives and destruction of barberry plants (W. Bushnell 1984). These receptors of various classes are encoded by stem rust genes (*Sr*) and were deployed as single genes in cultivated wheat. However, this resistance did not last long. A severe stem rust outbreak on wheat carrying the once effective *Sr31* resistance gene was observed in the 1998/1999 wheat growing season in Uganda, East Africa (Pretorius et al. 2000). This *Pgt* was termed "Ug99" (Uganda 1999) and its physiological race as determined by the stem rust international differential set (North American nomenclature) to be TTKS (Pretorius et al. 2000). This race designation was expanded to TTKSK following the addition of a fifth differential set containing monogenic lines carrying *Sr24*, *Sr31*, *Sr38*, and *SrMcN* in order to determine the pathotype of isolates within Ug99 group that had emerged with virulence to *Sr24* and *Sr38* in addition to *Sr31* (Jin et al. 2008). The virulence of the Ug99 group continued to evolve rapidly and there are 13 known variants at present which have been identified in 13 countries (**Table 1**).

Table 1.3 | *Puccinia graminis* f. sp. *tritici* races within the Ug99 group that have been identified in Africa and the Middle East.

Race	Main virulence (+) /Avirulence (-)	When Identified	Places Identified
TTKSK	+*Sr31*	1999	Uganda (1998/9), Kenya (2001), Ethiopia (2003), Sudan (2006), Yemen (2006), Iran (2007), Tanzania (2009), Eritrea (2012), Rwanda (2014), Egypt (2014)
TTKSF	-*Sr31*	2000	South Africa (2000), Zimbabwe (2009), Uganda (2012)
TTKST	+*Sr31*, +*Sr24*	2006	Kenya (2006), Tanzania (2009), Eritrea (2010), Uganda (2012), Egypt (2014), Rwanda (2014)
TTTSK	+*Sr31*, +*Sr36*	2007	Kenya (2007), Tanzania (2009), Ethiopia (2010), Uganda (2012), Rwanda (2014)
TTKSP	-*Sr31*, +*Sr24*	2007	South Africa (2007)
PTKSK	+*Sr31*, -*Sr21*	2007	[Uganda (1998/9)?], Kenya (2009), Ethiopía (2007), Yemen (2009), South Africa (2017)
PTKST	+*Sr31*, +*Sr24*, -*Sr21*	2008	Ethiopia (2007), Kenya (2008), South Africa (2009), Eritrea (2010), Mozambique (2010), Zimbabwe (2010)
TTKSF	-*Sr31*, +*Sr9h*	2012	South Africa (2010), Zimbabwe (2010)
TTKTT	+*Sr31*, +*Sr24*, +*SrTmp*	2015	Kenya (2014)
TTKTK	+*Sr31*, +*SrTmp*	2015	Kenya (2014), Egypt (2014), Eritrea (2014), Rwanda (2014), Uganda (2014)
TTHSK	+*Sr31*, -Sr30	2015	Kenya (2014)
PTKTK	+*Sr31*, -*Sr21*, +*SrTmp*	2015	Kenya (2014)
TTHST	+*Sr31*, -*Sr30*, +*Sr24*	2015	Kenya (2013)

Adapted from (Rusttracker 2017)

Recently, in 2017, race PTKSK was identified in South Africa (Terefe et al. 2019) after being identified in Zimbabwe seven years earlier (Pretorius et al. 2012). Races with combined virulence against *Sr31* and *Sr24* are continuing to spread. In addition, acquired virulence against *SrTmp* by TTKTT, TTKTK, and TKTK was detected in Kenya. Among these, TTKTK spread the fastest to countries such as Uganda, Rwanda, Egypt and Eritrea (Patpour et al. 2016).

It was Norman E. Borlaug who warned of the danger posed by Ug99 to food security. As a result, the Global Rust Initiative–now known as the Borlaug Global Rust Initiative–was formed in 2008 by the Consultative Group for International Agricultural Research organisations;

Indian Council of Agricultural Research (ICAR), International Center for Agricultural Research in the Dry Areas (ICARDA) and CIMMYT as well as the FAO (Food and Agriculture Organisation) and Cornell University in 2008 (BGRI, 2019). Research in rust disease control that had been neglected for nearly half a century became urgent as top wheat producing countries prepared for what was believed to be the inevitable appearance of the Ug99 race. Pardey *et al.*, 2013 predicted that a global investment of at least US$ 15 million per year in stem rust research was required to keep the disease at bay.

The establishment of a global rust monitoring system because of the threat of Ug99 led to large scale detection of various stem rust races. The origin of these races is not known. It has been suggested that they could have been incursions from other areas, or they were endemic to these areas but had remained undetectable and only became prevalent under conducive conditions (Olivera *et al.*, 2015). Among these were RRTTF and TKTTF (Table 1.4) which were distributed across East Africa, the Middle East, and South Asia (Singh et al. 2015). In 2013 and 2014, Ethiopia, the largest producer of wheat in sub-Saharan Africa (Negassa et al. 2013), experienced losses close to 100% due to stem rust epidemics over an area of approximately 10 000 ha (Olivera *et al.*, 2015). Of the 41 isolates that were analysed from the collection of 2013, the predominant race was TKTTF (29) followed by six isolates of TTKSK, then RRTTF (3) and JRCQC (3) (Olivera *et al.*, 2015). The predominance of TKTTF isolates could be attributed to the popularity of a cultivar called Digalu which was resistant to Ug99 due to carrying *SrTmp* as well as being resistant to *Pst*. The TKTTF race, however, was virulent against *SrTmp* carried by the Digalu cultivar and thus this group of isolates was called the Digalu (Singh et al. 2015). However, there are now Ug99 races (TTKTK and PTKTK) with virulence against *SrTmp* (Table 1.4) (Patpour *et al.*, 2016). The Digalu group of races is distinct from the Ug99 group because of avirulence on *Sr31* and *Sr11* (Olivera *et al.*, 2015). In Turkey, this race had been present from the 1990s and is still predominant (Mert et al. 2012). The identification of TKTTF was also confirmed in Iran (2010), Ethiopia (2012), Lebanon (2012), Egypt (2013), Georgia (2014), Eritrea, Azerbaijan and Yemen (Olivera *et al.*, 2015). Across Europe, *Pgt* variants were detected during widespread outbreaks in Turkey (2005-2013), Germany (2013), Sweden (2014), Denmark (2013) and Italy with the largest outbreak (20,000 ha) in 2016 (Olivera *et al.*, 2015; Olivera *et al.*, 2017; Lewis *et al.*, 2018; Patpour *et al.*, 2020). Isolated incidences also occurred in the UK and Ireland in breeding trial plots where susceptible plants were grown

(Lewis et al., 2018; Farmers Guardian, 2020). In the case of Ireland, occurrence was on a plot for a herbicide free trial (Farmers Guardian, 2020).

The *Pgt* race of the isolate(s) from Ireland has not yet been determined. Genetic analyses showed that the isolates in Turkey, Sweden, Denmark and Ethiopia were closely related to the TKTTF (Digalu) race and this was also confirmed by phenotypic analysis using the stem rust international differential set (Olivera *et al.*, 2015; Lewis *et al.*, 2018). Some of the German and Danish isolates differed from Digalu by having virulence on *Sr33*, *Sr7a*, *SrTt-3* and *Sr45* (Olivera *et al.*, 2015). The *Pgt* race responsible for the outbreaks in Italy was not related to TKTTF but TTRTF which is prevalent in Georgia where a sexually reproducing population is present (Olivera et al. 2019). In 2015 - 2017 stem rust was the major disease found on wheat in northern Kazakhstan and West Siberia whilst in 2016, 1 million hectares of wheat were affected by a stem rust epidemic in Russia (Rsaliyev and Rsaliyev 2018).

Table 1.4 | ***Puccinia graminis* f. sp. *tritici* races within the "Digalu" group (TKTTF) that have been identified in Africa, Europe and the Middle East.**

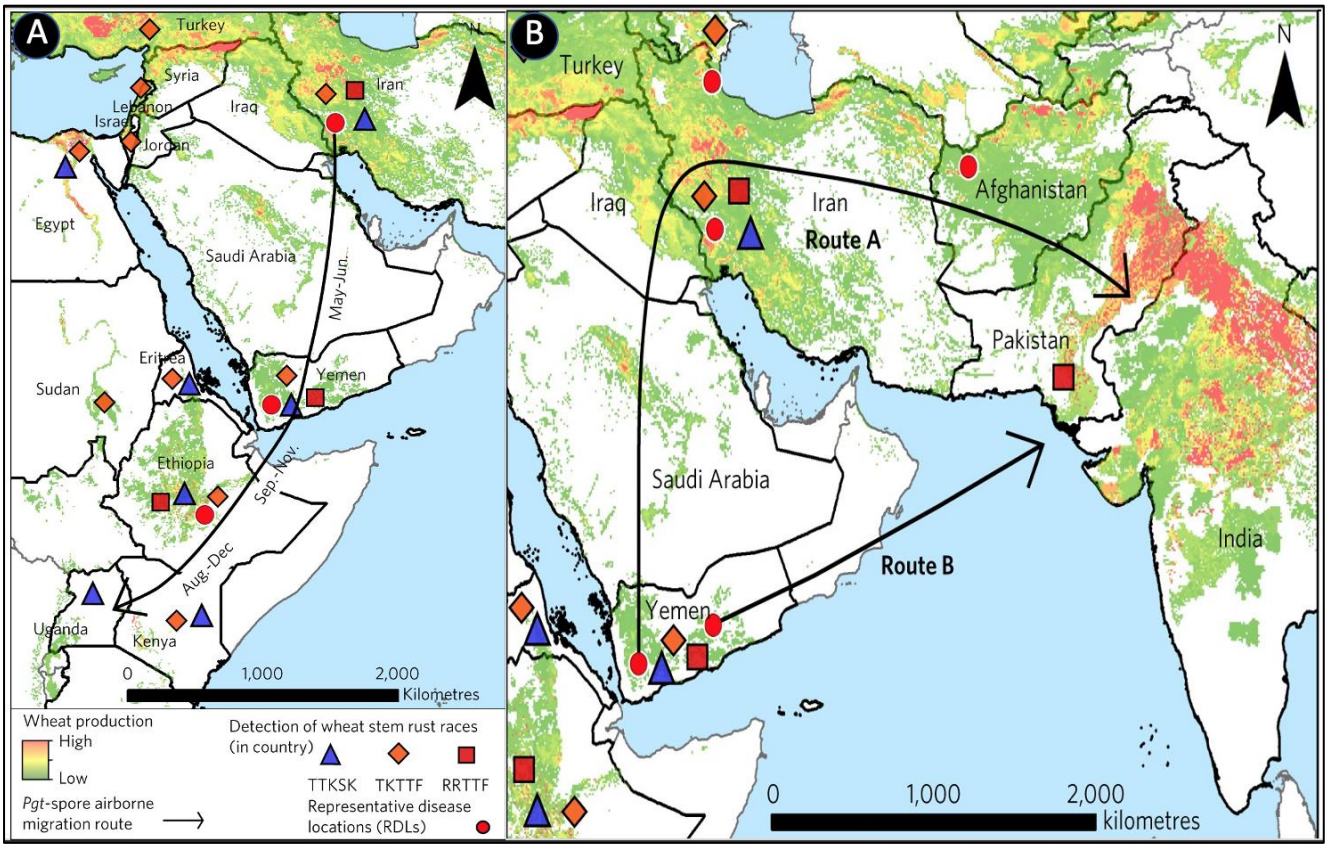
Race	Main virulence (+) /Avirulence (-)	When Identified	Places Identified
TKTTF	-*Sr31*, +*Sr33*	2013-15	Israel (2012), Ethiopia (2012), Sudan (2012), Egypt (2013), Turkey (2013), Yemen (2013), Denmark (2013), United Kingdom (2013), Germany (2013/4), Kenya (2014), Tanzania (2016), Sweden (2014)
PKPTF	-*Sr31*, -*Sr21*, +*Sr36*, -*Sr9b*	2013-4	Germany (2013), Georgia (2014)
TKKTP	+*Sr24*	2013	Germany (2013)
TKPTF	+*Sr31*, +*Sr36*	2013	Germany (2013)
TKFTF	-Sr36,	2013-15	Georgia (2013-5)

(Olivera et al. 2015; Olivera et al. 2017; Lewis et al. 2018; Olivera et al. 2019)

Climate change is expected to increase the incidence of *Pgt* in the UK and other European countries by the year 2050 as conditions become more favourable for the pathogen which is adapted to warmer climates (Juroszek and Tiedemann 2013). Currently, the late occurrence of *Pgt* in the wheat season as well as the heavy use of fungicides prevent significant damage. This however has begun to change. The stem rust race Ug99 is adapted to a lower temperature optimum than other races (West et al. 2012). With the presence of an endemic *Pgt* population as conditions become favourable, fungicide resistance could also rise as reported for pathogens like *Zymoseptoria tritici*, *Venturia inaequalis* and *Botrytis cinerea* (Schnabel and Jones 2000; Plesken et al. 2015; Zwiers et al. 2003). Diseases and pests can evolve and adapt to new environments resulting in changes in their geographical range due to changes in timing of pathogen growth stages with wheat and natural competitors (Chakraborty and Newton 2011). However, knowledge on how the incidence and distribution of wheat diseases will be affected by climate change is uncertain. Higher temperatures during winter and the cropping periods are expected to increase infectivity and sporulation of fungal pathogens of above-ground plant parts (Chakraborty and Newton 2011). Temperatures for growth of *Pgt* and its main host ranges between 17°C and 32 ^{o}C with minimums of 2 ^{o}C and 5 ^{o}C for spore germination and sporulation, respectively (Singh et al. 2002; Beddow *et al.*, 2013). The combination of temperature and water availability is an important factor in fungal infection. Infection and sporulation require high relative humidity which normally occurs during night conditions when dew forms and thus night temperature is important (Juroszek and Tiedemann 2013). Nocturnal temperatures have been predicted to rise more significantly than diurnal temperatures (Harvell 2002). Thus, global warming may increase the incidence of foliar fungi. Polycyclic pathogens can go through a greater number of generations during the growing season due to extended growing periods and rapid development because of warmer temperatures (Harvell, 2002).

The spread of Ug99, Digalu and other race groups over large geographical areas has been linked to long distance wind dispersal of inoculum (Fig 2) (Meyer et al. 2017a; Corredor-Moreno and Saunders 2020). Large scale monocropping of susceptible cultivars under environmental conditions suitable for pathogen growth enables spore producing pathogens like rusts to produce large quantities of urediniospores during a cropping season. Wind can then transmit these to nearby plants, or across great distances. Well known examples include

the importation of *Puccinia melanocephala* (sugarcane rust) into the Americas from Cameroon around 1978 and *Phakospora phachyrhizi* (Asian soyabean rust) into N. America from S. America and the movement of *Hemileia vastarix* (coffee leaf rust) from Angola to Brazil (Hovmøller et al. 2002, Visser *et al.*, 2019). More recently, microsatellite analysis and wind dispersal modelling showed how wheat stem rust isolates were introduced to Australia from Southern Africa as a result of a hurricane (Visser et al. 2019). Human-assisted pathogen dispersal because of human travel and agricultural trade have also enabled the introduction of pathogens despite the existence of phytosanitary regulations. Dispersal of the Ascomycete, *Mycosphaerella fijiensis*, the pathogen that causes black Sigatoka disease on banana, was due import of diseased planting material whilst wind dispersed ascospores locally (Brown and Hovmøller, 2002). More recently, another emerging serious pathogen of wheat, *Magnaporthe oryzae* pathotype *Triticum* (wheat blast) caused serious crop losses in Bangladesh. The DNA sequence of isolates of this pathogen from Bangladesh were nearly identical to those in Brazil (Islam et al. 2016) suggesting an incursion resulting from contaminated grain imports from Brazil. This poses a threat to the cultivation of wheat in neighbouring India via wind dispersal (Cruz and Valent 2017).



Reproduced from (Meyer et al. 2017)

Figure 1.4 | Possible routes of Puccinia graminis f. sp. tritici urediniospore transmission from the Middle East to East Africa (A) and within the Middle East (B)

With knowledge of how *Pgt* can spread over long distances, the question that remained was why the disease had resurfaced to cause large scale epidemics after over 50 years of successful breeding for resistance and barberry eradication that limited its presence to small, localised infections. The highlands of East Africa are regarded as a key region for rust epidemics and possibly the development of new infectious races (Negassa et al. 2013). The climate in the region is conducive for continuous cultivation of wheat hence providing a "green bridge", which is a continued source for stem rust inoculum which can spread to other wheat growing areas when their wheat producing seasons start (Negassa et al. 2013). In addition, there is the presence of *Berberis holstii* –a barberry species susceptible to rust– across much of East Africa (GBIF 2017). This could explain why new variants arose since *Pgt* reproduces sexually on this alternate host resulting in the emergence of virulent variants that are predominantly related to the parent race (Johnson 1954; Singh et al. 2006). However, this does not explain why new variants can appear where there is no sexually reproducing

population as has been the case in other areas. *Pgt* was introduced into Australia because of European emigration in the 18th century.

Even though sexual recombination has been eliminated by barberry eradication, the *Pgt* population has developed more than 100 pathotypes from the ancestral isolates which were categorised into four lineages by random amplified polymorphic DNA markers as well as isozyme analysis (Burdon and Silk 1997). This was seen as a result of somatic recombination and exchange, random mutations and selection due to development of cultivars with various resistances (Burdon and Silk 1997). With the advent of whole genome sequencing and analysis, evidence supporting the role of somatic hybridisation in the emergence of Ug99 and not sexual recombination was provided (Li et al. 2019). Somatic hybridization is a parasexual process that was first identified in *Aspergillus nidulans*. It involves hyphal fusion (anastomosis), reassortment (heterokaryosis) and fusion of unrelated haploid nuclei in the vegetative cells (diploidization), replication of these diploid nuclei, mitotic crossing over, and subsequent non-sexual haploidization of the diploid nuclei (Park and Wellings 2012).

By generation of a fully phased genome assembly and using DNA proximity analysis, it was shown that Ug99 has one of its haploid nuclei, termed haplotype A, similar to that of the Australian isolate *Pg21-0* with only 0.5% variation (Li et al. 2019). Variation was observed in the second haploid nuclei for Ug99 and *Pgt-21-0* (haplotype B and C respectively) (Li et al. 2019). This supports earlier propositions that TTKFS or PTKSF which are avirulent on *Sr31* and were present in East Africa might have been the progenitor of TTKSK through stepwise mutation (Singh et al. 2015). The acquisition of new virulence against other *Sr* genes by TTKSK has followed a similar pattern (Singh et al. 2015).

1.6. Control of wheat stem rust disease.

The demonstration that the Mendelian inheritance of disease resistance working on *Pst* resistance in wheat presented an opportunity to introduce variation to wheat and other crops to combat crop diseases (Bushnell 1984). Conventional breeding for stem rust control had significant success during the green revolution after introducing genes such as *Sr2*, *Sr24* and *Sr31* into cultivars (Section 1.3). The resistance conferred by *Sr31 d*erived

from rye was widely deployed and broken down nearly 50 years after its first introduction (Pretorius et al. 2000; Jin et al. 2008). Both *R* and APR genes are introduced into elite cultivars via crossing with other wheat lines or with wheat wild relatives. However, this is a lengthy process, and the resultant cultivars carry them as single genes. In the case of *R* genes this resistance is broken down due to the emergence of virulent isolates. Hence, multiple *R* genes need to be pyramided together with APR genes to provide more durable resistance.

1.6.1. The cloning and stacking of resistance genes in crops for durable resistance.

The introduction of multiple disease resistance genes is widely considered to potentially result in durable resistance in crops. Cultivars with pyramided genes can be produced by conventional crossing. Lines with durable resistance to *Pgt* race TTKSK, *Pt* and Fusarium head blight (FHB pyramided lines with multiple resistance genes were selected from a population of 68 doubled haploids that was a result of crossing the hybrid 'AC Cadillac' (*SrCad*, *Lr34*)/'Carberry' (*Lr34*, *Fhb1*) with 'RL5405' (*Sr33*)/'Carberry' (*Lr34*, *Fhb1*). Lines carrying the four genes *SrCad*, *Lr34*, *Fhb1* and *Sr33* were identified by marker assisted selection (Zhang et al. 2019). The stem rust resistance genes *Rpg1* and *Rpg4* were pyramided in barley via crossing and marker assisted selection (Sharma Poudel et al. 2018). Developing such lines can, however, present challenges as described previously. Pyramided genes can also segregate when transferring them to other cultivars.

To circumvent the limitations of cost, compatibility and linkage drag associated with introgression of effective *Sr* genes from wild relatives, multiple strategies to identify and clone genes (Table 2) have been devised over the years to enable their deployment in wheat and barley as transgenic stacks. The major advantage of the use of *R* gene stacks is the durable resistance when the individual genes have different avirulence effector specificities (Vleeshouwers and Oliver 2014). Cultivars with multiple traits can be developed where cultivars carrying single transgenes can be crossed thus creating cultivars with multiple transgenes. Thus, pyramiding can occur by combining genetic transformation and crossing. In the case of maize, the stacked transgenic cultivar 12–5 × IE034 containing the *Bacillus thuringiensis* insecticidal crystal protein genes *cry1Ie* and *cry1Ab* in addition to the *G10-epsps* gene for glyphosate intolerant 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), was developed from crosses of transgenic maize 12–5 and IE034 (Wang et al. 2019). The wheat

stem rust resistance genes *Sr22, Sr33, Sr35 and Sr45* have been shown to also be effective in controlling stem rust in barley as transgenes thus opening up the opportunity stack *Sr* genes from wheat in other *Pgt* susceptible crops (Hatta et al. 2020).

The first successful demonstration of stacking multiple disease resistance genes at a single locus in wheat was conducted with the seedling resistance genes *Sr22*, *Sr35*, *Sr45*, *Sr46* and *Sr50* and the multi-pathogen adult plant rust resistance gene *Lr67/Yr46/Pm46/Ltn3/Sr55* in constructs containing either three, four, five or six genes (Luo et al. 2018). These were incorporated into the *T. aestivum* genome via Agrobacterium-mediated transformation resulting in up to six genes located at a single locus in the cultivar Fielder. Field trials of Fielder-derived transgenic plants carrying five genes were immune to stem rust infection (2Blades Foundation 2019).

A major objective of this PhD research was to contribute to this strategy of stacking *Sr* genes in cultivars of wheat by providing a means with which to individually test *Sr43* and *Sr45* when deployed in stacks. The cloning of additional *Sr* genes to cater for the diversity of virulences within the global *Pgt* population to enable the deployment of stacks that are effective against *Pgt* races prevalent within a geographical area is continuing. However, the testing of the function of each stacked *Sr* gene requires the cloning of the avirulence effector genes (*Avr*) that correspond to these *Sr* genes. The cloning of *Pgt Avr* genes has lagged that of the *Sr* genes. In the case of the stack deployed in cv. Fielder, only the *AvrSr35* and *AvrSr50* have been cloned (Salcedo et al. 2017; Chen et al. 2017).

Table 1.5 | Cloned genes effective in rust control.

Crop	Gene id	Product	Pathogens controlled	Reference
Wheat, wheat wild relatives and Rye	*Lr1*	NLR	*Pt*	(Cloutier et al. 2007)
	Lr10	NLR	*Pt*	(Feuillet et al. 2003)
	Lr13			
	Lr21	NLR	*Pt*	(Huang et al. 2003)
	Lr22a	NLR	*Pt*	(Thind et al. 2017)
	Lr34	ABC transporter	*Pt*, *Pst*, *Pgt*, *Bgt*, *Exserohilum turcicum* (maize)	(Krattinger et al. 2009)
	Lr67	Hexose transporter	*Pgt*, *Pst*, *Pt, Bgt*	(Moore et al. 2015)
	Sr13	NLR	*Pgt*	(Zhang et al. 2017)
	Sr21			(Chen et al. 2018)
	Sr22	NLR	*Pgt*	(Steuernagel et al. 2016)
	Sr26			(Zhang et al. 2020)
	Sr33	NLR	*Pgt*	(Periyannan et al. 2013)
	Sr35	NLR	*Pgt*	(Saintenac et al. 2013)
	Sr45	NLR	*Pgt*	(Steuernagel et al. 2016)
	Sr46	NLR	*Pgt*	(Arora et al. 2019)
	Sr50	NLR	*Pgt*	(Mago et al. 2015)
	Sr60	Tandem Kinase	*Pgt*	(Chen et al. 2020)
	Sr61	NLR	*Pgt*	(Zhang et al. 2020)
	SrTA1662	NLR	*Pgt*	(Arora et al. 2019)
	YrAS2388R	NLR	*Pst*	(Zhang et al. 2019)
	Yr5	NLR	*Pst*	(Marchal et al. 2018)
	Yr5b	NLR	*Pst*	(Marchal et al. 2018)
	Yr7	NLR	*Pst*	(Marchal et al. 2018)
	Yr10cg	NLR	*Pgt*	(Yuan et al. 2018)
	Yr15	RLK	*Pst*	(Klymiuk et al. 2018)
	Yr36	Kinase-START	*Pgt*	(Fu et al. 2009)
	YrU1	NLR	*Pgt*	(Wang et al. 2020)
Pigeon pea	*CcRpp1*	NLR	*Phakopsora pachyrhizi*	(Kawashima et al. 2016)
Maize	*Rp1_D*	NLR	*Puccinia sorghi*	(Collins et al. 1998)
	Rp3	NLR	*P. sorghi*	(Webb et al. 2002)
Barley	*Rpg1*	RLK	*Pgt*	(Brueggeman et al. 2002)
	Rpg5	NLR	*Pgt*	(Brueggeman et al. 2008)
	Rph1	NLR	*Pt*	(Dracatos et al. 2019)
	Rph15	NLR	*Pt*	(Chen et al. 2021)
	Rphq2	RLK	*Puccinia hordei-bulbosi*	(Wang et al. 2019)
	Rph22	RLK	*P. hordei*	(Wang et al. 2019)

Adapted and updated from Keller *et al*. (2018)

1.6.2. The use of crop mixtures.

Large scale wheat cultivation tends to be done as vast fields of single cultivars. This can lead to breakdown of resistance due to the application of selection pressure on the endemic pathogen population or incursion by virulent isolates as most cultivars carry single genes which provide strong resistance (Balesdent et al. 2013; Agrios 2005). Pathogens can overcome this resistance when they lose the corresponding avirulence effector (*Avr*). The cultivation of mixtures of varieties with different resistances can provide some measure of control. This has been shown for stripe rust of barley where inoculum was significantly decreased whilst the yield of susceptible cultivars improved due to cultivar mixing (Pradhanang and Sthapit 1995). Susceptible cultivars in crop mixtures slow down the evolution of virulence by providing "refuge" and this strategy has also been used in the control of lepidopteran pests in maize (Al-Adil and Al-Jassany, 1992; Storer et al., Head, 2012). The provision of refuges can allow for the reversion of pathogens to avirulence as in some cases loss of avirulence genes is associated with fitness cost. This could potentially be useful in controlling pathogens such as the ascomycete fungal pathogen *Verticillium albo-atrum* where the effector gene *Ave1*, which corresponds to the resistance gene *Ve1* in tomato, is required for full aggressiveness of Verticillium on tomato plants lacking *Ve1* (De Jonge et al. 2012). Another potential example at hand includes the effector gene *AvrLm4* from the oilseed rape pathogen *Leptosphaeria maculans*, the causal agent of phoma stem canker. Isolates lacking *AvrLm4* produced smaller infection lesions than those with *AvrLm4* and in the field. In the absence of cultivars with the corresponding *Lm4*, the proportion of isolates with the *Avr* increased (Huang et al. 2006). As knowledge of the *Avr* arsenal and their biological function increases, breeders can reintroduce defeated resistance genes after analysing the effector component of a prevailing pathogen population and also deploy mixtures of cultivars that have different *R* gene specificities (Vleeshouwers and Oliver 2014). There are limitations, however, to the approach of using cultivar mixtures. Cultivars have different end-use qualities and it can be difficult for farmers to harvest them separately in order to meet market requirements (Castro 2001). Farm operations can become complicated and costly when lines in the same field mature at different periods or have different cultivation requirements which can make mechanical operations difficult and cause reliance on labour (Castro 2001). The use of multi-lines, which are near-isogenic and differ mainly in the resistance gene they carry can

provide a solution to this. However, they do require a long time to develop whilst speed breeding can help reduce the time it takes (Muehlbauer et al. 1988; Watson et al. 2018). Between 6-15% of the area cultivated under wheat in the states of Washington, Oregon, and Kansas in the US is planted as mixtures (Faraji 2011).

1.6.3. The use of fungicides.

Fungicides can be applied to wheat to control rust diseases. Some fungicides like sterol biosynthesis inhibitors (SBIs) or demethylation inhibitors (DMIs) that inhibit the biosynthesis of sterols are highly effective.

Fungicides have varied efficacies in the eradication of control stem rust disease. The fungicide with the trade name Folicur® (Table 2) has the active ingredient tebuconazole (Bayer Crop Science 2020). It is a broad-spectrum systemic fungicide in the triazole group for winter and spring crops of bread wheat, barley, oats, rye, oilseed rape, field beans and linseed (Bayer Crop Science 2020). Folicur® was shown to be more effective than Triad® (triadimefon) or Impact® (flutriafol) in reducing stem rust disease and increasing yield (Loughman et al., 2005). This was also confirmed in another study where Folicur® and other various fungicide applications used lowered disease severity and higher yields compared to untreated check plots (Wanyera *et al.*, 2009). Other fungicides (Table 2) like AmistarXtra®280 SC (Azoxystrobin + Cyproconazole), Silvarcur® 375 EC (tebuconazole + triadimenol), and Orius® 25 EW (broad spectrum curative and preventive triazole) were more effective in decreasing disease severity whilst increasing yield (Wanyera *et al.*, 2009). Fungicides such as Stratego® 250 EC (Prothioconazole + Trifloxystrobin), Cotaf® 5 EC (Hexaconazole), Swing®Gold 250 EC (dimoxystrobin + epoxiconazole), Artea™ 330 EC (Cyproconazole), and Soprano® 250 EC (Epoxiconazole) were inconsistent (Wanyera *et al.*, 2009) despite being effective for other fungal pathogens such as leaf and stripe rust, fusarium head blight, and leaf rust, stripe rust, Septoria nodorum blotch and powdery mildew (Table 2).

In poorer countries where stem rust also happens to be more prevalent, the cost of application can be prohibitive for routine preventative sprays for rural farmers. Fungicides enter the environment as a result of spray drift and surface runoff from fields (Potter et al., 2014). Their residues can be found in produce, soil, water discharged from water treatment

plants, human urine and hair (Xuan *et al.*, 2017). Their toxicities have been studied and shown to have toxicity to the liver, male reproductive system and developmental toxicities induced by triazole fungicides mice and zebrafish (Mu et al. 2016; S. D. Hester et al. 2006; S. Hester et al. 2012). Tebuconazole, uniconazole, hexaconazole, penconazole, bitertanol were shown to inhibit CYP3A which is involved in the biotransformation of testosterone *in vitro* at environmentally feasible concentrations (Xuan *et al.*, 2017). In addition one study showed that tebuconazole (Folicur®) amplifies the toxic effect of low doses of the neonicotinoid insecticide thiacloprid and poses a risk to parasitic wasps that are used as biological control agents of insect pests (Willow et al. 2019). Other types of fungicides also have been shown to have a negative impact on non-target organisms. Widely used products like Rovral® 4F, a dicarboximide used to control *Botrytis* that inhibits triglyceride biosynthesis, and the strobilurin Pristine® (strobilurin) in orchard crops were found to disrupt the nest recognition of female bees (Artz and Pitts-Singer 2015). In 2019, the European Union which is the largest producer of wheat in the world banned the use of Chlorothalonil (Bravo®, Echo®, and Daconi®l) (Farmers Guardian 2019). Chlorothalonil is a carcinogen and poses a risk to humans, fish and amphibians (Arena et al. 2018). This fungicide was highly effective and had been widely used in wheat and barley production for over 50 years and was the cheapest option for disease control (Farmers Guardian 2019).

There is also the challenge of evolution of fungicide resistance by the pathogen due to the single site mode of action of modern fungicides. Plant pathogenic fungi like Venturia, Botrytis, Monilia and Penicillium have now developed resistance to benomyl® (benzimidazole) (Deising et al., 2008), while *Mycosphaerella fijiensis* has evolved resistance to strobilurin fungicides (Sierotzki et al. 2000). These challenges to fungicide use require that other approaches be employed as the reliance on a few fungicides with similar modes of action that are still considered to be safe poses a risk for evolution of pathogen resistance.

1.6.4. RNA interference in the control of wheat and barley diseases.

Naturally, plants and fungi can secrete short RNAs (sRNA) which can interact with each other in a phenomenon commonly known as cross-kingdom RNA interference (ck-RNAi) (Cai et al. 2018; Zhang et al. 2016). The level of importance of ck-RNAi is not fully understood. RNA silencing is a well conserved system that functions in most eukaryotes (Tuo Qi et al. 2019). RNA silencing typically involves small RNAs that are between 21 and 30 nucleotides (nt) long, which have the ability to regulate gene expression in a sequence dependent way (Tuo Qi et al. 2019). In Arabidopsis plant-originated sRNAs were delivered into *Botrytis cinerea* via small extracellular vesicles (Cai et al. 2018). Arabidopsis mutants with impaired vesicle trafficking were more susceptible to mould infection. The siRNA delivered to *B. cinerea* downregulated BC1G_08464 which encodes a suppressor of actin (SAC1)–like phosphoinositide phosphatase that is involved in pathogen virulence (Cai et al. 2018). The existence of Ck-RNAi can be partly explained by the existence of three proteins across eukaryotes that are necessary for RNAi (RNA interference) namely, an argonaute (AGO) protein, a dicer-like (DCL) protein, and RNA-dependent RNA polymerase (Schaefer et al. 2020).

The presence of this conserved machinery in eukaryotes presents opportunity for the application of RNAi in crop protection. The plant immune system utilises RNA silencing machinery, as a defence against viral pathogens called virus induced gene silencing (VIGS) (Tuo Qi et al. 2019). VIGS can be exploited to develop host-induced gene silencing (HIGS) technology targeting other plant pathogens such as fungi. HIGS is therefore an RNAi-based system where plants can be induced to express dsRNAs that are 21-30 nucleotides (Tuo Qi et al. 2019). The constructs for the dsRNA are designed to target specific genes in the pathogen to suppress growth or virulence using viral or agrobacterium vectors (Tuo Qi et al. 2019). For example, *Avra10* produced by the obligate biotroph *Blumeria graminis* f. sp. *hordei* (*Bgh*) is recognised by resistance gene *Mla10* (Ridout et al. 2006b). The transient expression of dsRNA (double-strand RNA) to silence transcripts of *Avra10* in planta as well as the 1,3-b-glucanosyltransferases *BgGTF1* and *BgGTF2* limited the development of the fungus (Nowara et al. 2010). Growth could be recovered by the expression of a synthetic *Avra10* with point mutations that made it not susceptible to silencing (Nowara et al. 2010).

The barley stripe mosaic virus (BSMV) vector has been used successfully to induce HIGS in wheat against rusts. Agrobacterium-mediated transient silencing of *Pgt*, *Pst*, *Pt* mitogen-activated protein kinase 1, cyclophilin, and calcineurin B resulted in reduced fungal biomass, emergence of uredinia and virulence (Panwar et al., 2013). Examples of other genes that have been silenced by BSMV-HIGS include protein kinase, small GTP-binding protein (*PsRan*) *PsRas1* and *PsRas2*, MADX-box transcription factor (*PstMCM1-1*) (Tuo Qi et al. 2019). The transcription factor *PstSTE12* and MAPKKK *PsKPP4* (Cheng et al. 2016, 2017; Zhu et al. 2018). Genes encoding for secreted protein effectors in *Pst* have also been silenced, such as *Pst_8713*, *PSTHa5a23*, *PEC6*, and Zn-only superoxide dismutase (*PsSOD1*) (Liu *et al.*, 2016; Liu *et al.*, 2016; Cheng *et al.*, 2017; Zhao *et al.*, 2018). Some of these genes are important for virulence and their silencing caused significant reduction in hyphal growth of the hyphae whilst *PsRan* and *PsSRPKL* knockdown results in abnormal phenotypes such as hypertrophy and small haustoria. The silencing of genes involved in the growth and development of *Pgt* offers an opportunity to add another layer of defence in the quest for durable rust control. The pathogen resistance due to the knockdown of effectors might be lost due to effector mutations and redundancy unless conserved effectors are targeted.

1.7. Plant immunity overview.

Plants have evolved molecular mechanisms that function as an inherent immune (basal) system that activates defence responses induced by receptors at two levels: those on the cell surface monitoring the intercellular space (apoplast), and then intracellular receptors that survey the cytoplasm, for invasion by pathogens and pests (Jonathan D.G. Jones and Dangl 2006). These mechanisms of plant disease resistance are controlled by genes (Agrios 2005). These will vary according to the plant species or cultivar as discussed previously for rust pathogens. These genes confer to plants the capability to identify and fight off invasion at different points during their attempt to colonise their potential host (Agrios 2005).

Some receptors in the plant immune system have structural features in common with that of animals and the study of these receptors in animals has benefitted research in plant immunity (Duxbury et al. 2020; Bentham et al. 2017). However, the major components of immunity in animals are immunoglobulins which are adaptive, and plants lacking these, rely solely on an innate immunity to recognise microbial pathogens and pests (Jones et al. 2016). Hence the

receptors involved in plant basal immunity are evolutionarily more vastly expanded than in animals (Meunier and Broz 2017).

Extracellular immune receptors detect conserved or secreted molecules of pathogens or pests, outside the host cell via extracellular domains (Jones and Dangl 2006). Upon recognition, responses to resist infection are triggered via their intracellular kinase domains (Kanyuka and Rudd 2019). Some of these receptors sense molecules produced as a result of damage due to pathogen and pest activity (Boutrot and Zipfel 2017). Immune receptors located within the cell detect signatures of invasion by pathogens or pests that are adapted to the host (van Wersch et al. 2020; Hogenhout and Bos 2011). These signatures are a wide range of molecules that are delivered into the host cells in attempt to promote successful infection and completion of the (Białas et al. 2018). Activation of plant innate immunity can be associated with localised cell death that constrains the spread of infection especially in the case of biotrophic pathogens like rusts (Greenberg 1997). However, some pathogens purposely trigger cell death and acquire nutrients saprophytically such as *Phytophthora infestans* in its necrotrophic growth phase. The fungal effector SnTox1 produced by *Phaeosphaeria nodorum*, is recognized by Snn1, a wall associated kinase (WAK) cell surface receptor from wheat resulting in susceptibility (Liu et al. 2012).

1.7.1. Pattern recognition receptors, the first layer in immunity.

This first component in plant immunity involves defence responses which are triggered by products of cell wall digestion (by hydrolytic enzymes secreted by invading pathogens referred to as *d*amage *a*ssociated *m*olecular *p*atterns (DAMPs) or by highly conserved pathogen components like lipopolysaccharides, chitin, glucans and flagellin which are called *p*athogen/ microbial *a*ssociated *m*olecular *p*atterns (P/MAMPs) (Jones and Dangl, 2006; Zipfel, 2009; Tang et al. 2017). This component in immune defence serves to discriminate between plant cells, and beneficial and pathogenic microorganisms (Kanyuka and Rudd 2019). The receptors involved in this are termed *p*attern *r*ecognition *r*eceptors (PRRs). The immune response that is triggered by (PRRs) is termed PAMP triggered immunity (PTI) (Dodds and Rathjen 2010; Bentham et al. 2020).

There are two main types of PRRs in plants namely, *l*eucine-*r*ich *r*epeat (LRR) *r*eceptor-*l*ike *k*inases (RLKs) and membrane bound LRR-*r*eceptor-*l*ike *p*roteins (RLPs), which have significant similarities to LRR containing Toll-like receptors (TLRs) in mammal immunity (Jones and Takemoto 2004). LRR-RLKs are transmembrane proteins composed of an extracellular LRR receptor domain (eLRR), a transmembrane domain and a Ser/Thr protein kinase domain located inside the cell below the cell membrane (Zipfel 2008). RLPs are similar to RLKs in structure but lack a cytoplasmic kinase domain (Schwessinger and Zipfel 2008). Other variations in PRR can be a kinase domain(s) without an extracellular domain (Zipfel 2008). Animals do have PRRs to recognize pathogen MAMPs or molecules during infection resulting in an inflammatory response (Chen and Nuñez 2010). PRRs are not only involved in plant defence but also regulation of growth, reproduction, development, symbiotic relationships as well as abiotic stress tolerance (Breiden and Simon 2016).

PTI usually does not involve cell death upon detection of MAMPs or DAMPs. Typical PTI activity include the rapid generation of reactive oxygen species (ROS) which are a combination of •OH, H_2O_2 and O_2^- (ie oxidative burst) and the expression of immune-related responses such as antimicrobial compounds elevated salicylic acid (SA) and Ca_2^+ levels (Boller and Felix 2009; Klessig, Choi, and Dempsey 2018). After recognition, defence responses remain in a heightened state and can provide protection against infection by other pathogens which is termed systemic acquired resistance (SAR) (Klessig, Choi, and Dempsey 2018). It has been shown that exogenous application of, SA and PAMPS can potentially be used to induce SAR in plant protection as the response to infection by a primed immune system is stronger and faster (Klessig et al. 2018).

The gene *Sr60* which is designated *WHEAT TANDEM KINASE 2* (*WTK2*) was cloned from *T. monococcum* and encodes a PRR with two kinase domains without transmembrane or eLRR domains (Chen et al. 2020). *WTK2* provided resistance to *Pgt* when it was expressed in hexaploidy wheat where it significantly reduced pustule size. The *WHEAT TANDEM KINASE 1/ Yr15* encodes an non-arginine-aspartate (non-RD) kinase (Klymiuk et al. 2018). Non RD kinases are involved in the recognition of PAMPs (Dardick, Schwessinger, and Ronald 2012).

The knowledge of the structure of PRRs and that they recognise PAMPs thus conferring resistance which a broad range of pathogens cannot easily overcome makes them ideal for cloning and deployment in crops. Recently, Lin *et al.* (2020) developed a novel method for RLP/K enrichment sequencing. This follows the same approach like *R* gene *en*richment *seq*uencing (RenSeq) (Witek et al. 2016) and its variations like MutRenSeq and AgRenSeq which were developed for identification of intracellular receptors (Steuernagel et al. 2016; Arora et al. 2019). Using *P. infestans* as a model, they first screened potato wild *Solanum* relatives that recognised the effectors INF1 and SCR74, Nep-1 like proteins that are secreted in the apoplast and are conserved across bacteria, fungi and oomycetes (Oome et al. 2014). Following this, they conducted RLP/KSeq, to map their receptors using bulked segregant analysis and then fine-mapped the locus for a *G-LecRK* receptor gene for SCR74 (Lin et al. 2020). PRRs determine pathogen host specificity.

Chitin, which makes up fungal cell walls is a major PAMP. *CERK1* (chitin elicitor receptor kinase 1) in *Arabidopsis thaliana* perceives chitin and signals to initiate defences alone whereas *CEBiP* (chitin elicitor binding protein) in rice is required in addition to *CERK1* to achieve this (Lee et al. 2014; Miya et al. 2007). Arabidopsis *CERK1* carries three lysin motifs (LysM) that are involved in chitin-binding in the apoplast (Miya et al. 2007). Both *CERK1* and *CEBiP* have wheat orthologues (Lee et al. 2014). The hemibiotrophic *Zymoseptoria tritici* avoids detection by these PRRs by secreting the effector *Mg3LysM,* which is a homologue effector *Ecp6* from the biotrophic pathogen *Cladosporium fulvum,* and functions in tightly binding to fungal chitin thus sequestering it from host receptors and chitinases (Lee et al. 2014; Sánchez-Vallet et al. 2020; Bolton et al. 2008).

In the recognition of Bacterial PAMPs, flagellin epitope flg22 and the most abundant bacterial protein, elongation factor TU (EF-Tu) or its epitope efl18 are recognised by RLKs FLS2 and EFR (Kunze et al. 2004; Zipfel et al. 2006). EF-Tu is recognised by the receptor kinase EFR and this has been shown to reduce the efficiency of *Agrobacterium*-mediated transformation in Arabidopsis (Zipfel et al. 2006). The rice extracellular receptor Xa21 binds RaxX21-sY, a protein form *Xanthomonas oryzae* pv oryzae (Pruitt et al. 2015). Inter species transfer of Xa21 from rice to banana conferred resistance to *Xanthomonas campestris* pv. *Musacearum*, the causative agent of Banana Xanthomonas/ bacterial Wilt (Tripathi et al. 2014).

PRRs can also recognise pathogen secreted effectors resulting in PTI or reduced virulence. *Pseudomonas syringae* pv. *tomato* causes bacterial speck of tomato and secretes a type III secretion system (T3SS) effector AvrPto that directly interacts with the kinase Pto resulting in resistance (Tang et al. 1996). *P syringae* secretes effector AvrPtoB to target the Arabidopsis RLK LecRK-IX.2 in order to degrade it and suppress PTI (Xu et al. 2020). Conversely, LecRK-IX.2 phosphorylates AvrPtoB thus preventing its self-association which is required for the degradation of LecRK-IX.2 and thus restoring AvrPtoB recognition and PTI (Xu *et al.*, 2020). The wheat wall associated kinase Stb6 binds to AvrStb6 of *Z. tritici* (Saintenac et al. 2018; Zhong et al. 2017). In the well-studied tomato fungal pathogen *Cladosporium fulvum*, extracellular avirulence effectors Avr2, Avr4, Avr4E, Avr9 and effectors are recognised by Cf-2, Cf-4, Cf-4E and Cf-9 (Stergiopoulos and De Wit 2009; Dixon et al. 1996; Thomas et al. 1997; Wulff et al. 2009). Avr2 binds to Rcr3 and PIP1 which are enriched in response to pathogen attack and SA with PIP1 accumulating at higher levels than Rcr3. However, Cf-2 recognises the protease inhibitor Avr2 through its activity when it binds to the protease Rcr3 suggesting that Rcr3 might serve as a decoy for PIP1 (Van Der Hoorn and Kamoun 2008). Rcr3 inhibition has been shown not to contribute to virulence.

In some cases, pattern recognition controls non-adapted pathogens and this is called non-host resistance, which is the immunity of a species of plants to entire complement of races or strains of a possible pathogen species (Mysore and Ryu 2004). Because of this kind resistance, pathogens that are successfully adapted to a host secreted several effectors act to suppress PTI thus aiding host colonization. The Arabidopsis genome contains 610 LRR-RLKs with diverse functions ranging from disease resistance to plant development (Lehti-Shiu et al. 2009). The PRR EFR in Arabidopsis recognises the elf18 protein component of bacterial flagella. Transfer of this gene outside of the Brassica family to tobacco and tomato plants (Solanaceae) lead to a pathogen-dependent induction of the hypersensitive response HR and concomitant disease resistance to a wide range of bacterial pathogens (Lorang et al. 2007). This indicates the potential of interspecies transfer of PRR genes for disease control (Wulff et al. 2011). Wang *et al.* (2019) cloned two lectin RLKs, *Rphq2* and *Rph22,* that confer non-host resistance to leaf rust adapted to wild barley or cultivated barley respectively. These genes were cloned via map-based cloning from cultivated (*Hordeum vulgare*) and wild barley (*H. bulbosum*)

respectively (Wang *et al.*, 2019). *Rphq2* from cultivated barley exhibited a stronger resistance phenotype to the non-adapted leaf rust of wild bulbous barley (*P. hordei-bulbosi*) whilst the resistance was significantly lower when infected by leaf rust adapted to cultivated barley (*P. hordei*). The resistance of RLK *Rph22* native to wild bulbous barley was significantly stronger to non-adapted barley leaf rust than for leaf rust adapted to it. (Wang *et al.*, 2019). This makes the cloning of PRRs involved in non-host resistance attractive for use in the control of pathogens adapted to cultivated crops. However, identifying the components of non-host resistance is complex since it involves many receptors. If successful, the use of such PRRs in cultivated crops can result in "host jumps" where previously non-adapted pathogens can evolve to overcome the resistance resulting in the emergence of new pathogens.

1.7.2. Evolution of cell surface receptors.

Studies of RLKs in the Arabidopsis genome show that they belong to one of the largest gene families. Comparative analysis of four fungal, six animal, and two *Plasmodium* sp. genomes revealed that only fungal genomes did not have them suggesting an ancient origin for the family which was subsequently expanded only in plants (Lehti-Shiu et al. 2009). Tandem and segmental duplications are the major processes for this. Duplication of RLKs provides for novel PAMP recognition. The extracellular domains are under accelerated evolution to recognize novel PAMPs (Fischer et al. 2016). Gene duplication of RLKs involved in growth and development is rare unlike that of genes involved in defence response to biotic stress (Lehti-Shiu and Shiu 2012). Recognition of flg22 occurs across most spermatophytes (Boller and Felix 2009; Albert et al. 2010). Orthologues of *FLS2* have been characterised in *A. thaliana*, *Nicotiana benthamiana*, *Solanum lycopersicum*, *Vitis vinifera* and *O. sativa* thus flg22/ FLS2 interaction arose earlier than angiosperms (Hann and Rathjen 2007; Robatzek et al. 2007; Macho and Zipfel 2014). The slow evolution of PAMPs means PRRs evolve under negative selection to maintain their perception (Vetter et al. 2012). Analysis of RLK genes in angiosperms provide evidence that most of them undergo negative selection over long time scales (Fischer et al. 2016). Conversely, evolution of PAMPs such as flagellin flg22 and flgII-28 epitopes within species also occurs, and different these alleles induce different immune responses (Cai et al. 2011). Thus, PRRs evolve to efficiently bind to PAMP alleles. Where indirect recognition occurs such as that of *C. fulvum* Avr2 by receptor Cf-2; Rcr3 evolves by

duplication, rapid gene conversion and balancing selection in the wild tomato species *Solanum peruvianum* whilst *Cf-2* undergoes balancing selection in the wild tomato species *Solanum pimpinellifolium* (Caicedo and Schaal 2004; Hörger et al. 2012). Based on this growing evidence, the teasing apart of non-host resistance machinery and the subsequent interspecies transfer of individual PRRs for durable pathogen resistance could pose a risk of host jumps. Pathogens adapted on crops carrying the introduced receptor(s) can evolve virulence and become pests on the native sources of the genes or there can be emergence of new pathogens on the recipient of the alien PRR (Ayliffe and Sørensen 2019).

1.7.3. NLRs serve as intracellular immune receptors.

Since some pathogens evade cell surface receptors by masking or evolving PAMPs and suppressing PTI, plants have evolved intracellular immune receptors which serve as an additional layer of immunity (Jones and Dangl, 2006; Win *et al.*, 2012). Resistance (*R*) genes mostly code for NLRs which are made up of a nucleotide binding (NB) domain and a leucine rich repeat domain (Jones et al., 2016). NLR proteins were first discovered in plants and thereafter in humans and animals (Ting et al. 2008). In plants and animals, NLRs function in pathogen detection and developed independently through convergent evolution (Jacob et al., 2013). Animal NLRs work just like PRRs described previously detecting PAMPs or DAMPS thus initiating immune responses such as inflammation (Jacob et al., 2013).

In plants NLR genes subscribe to the gene-for-gene hypothesis which specifies that for every *R* gene in the host there is a corresponding avirulence (*Avr*) effector gene in the pathogen (Bushnell 1984). Oort independently observed this gene-for-gene relationship (now understood to be *R-Avr* interaction) while working *with Ustilago tritici* (wheat loose smut) around the same time as the more widely recognised work by Flor with the *M. lini* (flax rust)-flax pathosystem (Oort 1944; Flor 1942). Recognition of avirulence effector genes is normally associated with a strong immune response which is characterised by death of the invaded cells and other downstream responses classified as effector triggered immunity (ETI) (Jones and Dangl, 2006; Dodds and Rathjen, 2010). This gene-for-gene interaction forms the basis of the hypothesis of this PhD research where we expect mutations in *Pgt Avr* genes will result in gain of virulence by the pathogen towards their corresponding *R* genes. A pathogen is able to

overcome resistance if a mutation occurs in the Avr gene resulting in the corresponding *R* gene being unable to detect it (Ellis et al. 2014).

1.7.4. NLR structure.

In plants, NLR proteins are classified into three diverged groups according to the type of their N-terminal domains (Collier *et al.*, 2011; Shao *et al.*, 2014)

- Toll/Interleukin-1 receptor/Resistance (TIR) NLRs (TNLs),
- coiled-coil (CC), NLRs (CNLs),
- and RPW8-like coiled-coil domain NLRs (CCR-NLs or RNLs)

Plant NLRs comprise a C-terminal leucine-rich repeat (LRR) domain, a central nucleotide-binding domain known as the NB-ARC domain (nucleotide-binding adaptor shared by Apaf-1, Resistance proteins, and CED-4) and a variable N-terminal module, which can either be a TIR (Toll/interleukin-1receptor/resistance), CC (coiled-coil) domain, or a RPW8-like CC domain (CC-RPW8) (Van Der Biezen and Jones 1998; Duxbury et al. 2016). Transient expression have shown that the N-terminal domains can initiate cell death autonomously, and in the absence of an effector (Bentham et al. 2020). The NB-ARC proteins belong to a subgroup in the STAND family (signal transduction ATPases with numerous domains) (Qi and Innes 2013). They function as molecular switches regulating several processes such as immunity and apoptosis (van Ooijen et al. 2008). This was first established in loss-of-function experiments using the CNL from tomato *I-2* that confers resistance to *F. oxysporum* (Tameling et al. 2002). PRRs and NLRs both have LRR domains which are involved in pathogen recognition. For NLRs, the LRR domain recognises effectors and they could also function in the autoinhibition of the receptor (Bentham et al. 2017; Dodds et al. 2004).

1.7.5. How NLRs signal.

NLRs and the effectors they detect can localize to varied locations in the cell like the cytoplasm, nucleus, plasma membrane, tonoplast, and endoplasmic reticulum (Lolle et al. 2020). The CNL MLA10 and TNL RPS4 in barley and Arabidopsis respectively, are required in both the nucleus and cytoplasm for full resistance. The Arabidopsis CNL RPM1 localises with

the plasma membrane. What is unknown is how despite their various locations, NLRs still trigger similar defence responses.

NLR activity is controlled by processes such as self-inhibition, dimerization or oligomerization, epigenetic and transcriptional regulation, alternative splicing and proteasome- mediated degradation (Lolle et al. 2020). It has been widely believed that when inactive, NLRs are tightly folded and bound to ADP whilst detection of effectors results in structure changes that enable ATP binding and other structural formations. Recently the first crystal structures of a plant NLR, ZAR1 (HOPZ-ACTIVATED RESISTANCE 1) complex in its inactive, intermediate and activated states were established (Wang et al. 2019). ZAR1 self-associates in its inactive state via inter-domain interactions and its LRR domain interacts with the pseudokinase RKS1. Upon activation, PBL2 recruits binds to RKS1 resulting in strutural changes in the NBS domain of ZAR1's which releases ADP and forms of a ZAR1-RSK1-PBL2 complex which is seen as a primed intermediate state. dATP or ATP binding and the subsequent changes within the NBS domain, mediates oligomerization of the complex into wheel-like pentamer, termed the resistosome. Upon oligomerization, the N-terminal ZAR1 CC domains form a protruding funnel-like structure which resembles pore-forming toxins. These N-terminal α-helices of the CC domain are required for membrane association and signalling when ZAR1 is activated. In animals, pathogen detection causes NLRs to undergo changes forming inflammasomes and apoptosomes that trigger cell death. Thus, ATP binding, oligomerization and cell death induction seems to be a feature of NLR activation (Lolle et al. 2020).

The elucidation of the resistosome structure led to new understanding in how NLRs are activated and how downstream downstream signalling occurs. The membrane disruption previously described could be recognised by PRRs as DAMPs causing signalling that increases immune responses (Wang et al. 2019a). Alternatively, CNL resistosomes could form selective ion channels and transport signalling ions, such as Ca^{2+}. This requires additional mechanisms to control ion selectivity (Lolle et al. 2020). Recently, the RPW8 domain-containing "helper" NLR (RNL) called N REQUIREMENT GENE 1.1 (NRG1.1) was discovered to have an N-terminal conformation like that ZAR1 in its resting state and the animal MLKL (MIXED-LINEAGE KINASE-LIKE) cation channel (Jacob et al. 2021). NRG1.1 oligomerized when activated and enriched in

plasma membrane resulting in cytoplasmic Ca^{2+} influx in both plant and human cells (Jacob et al. 2021).

The localisation of NLRs to many cellular components leads to the question of whether they can form these structures in membranes.

Downstream responses from CNL receptors require the NDR1 locus, while TNL receptors require combinations of lipase-like proteins like EDS1 and SAG101 (Lolle et al. 2020). NDR1 is located at the plasma membrane and it facilitates plasma-membrane cell wall adhesions whilst also having similarity to abiotic stress response proteins (Lolle et al. 2020). TNLs lack CC domains and have been seen to require helper NLRs of the CNL class, including ADR1, NRG1 and NRCs (Wu et al. 2017). TNLs Roq1 and RPP1 need the helper NLR NRG1 (Qi et al. 2018). Deletion of 13aa from the N-terminal of the CC-domain of NRG1 blocks initiation of cell death (Lolle et al. 2020).

PTI and ETI are typically associated with ROS burst (Jones and Dangl 2006). In the extracellular space, ROS strengthens the plant cell wall, initiates cell wall depositions and is also a secondary signal required for immune responses (Kadota et al. 2015). In Arabidopsis, respiratory burst oxidase homolog D (RBOHD), is the primary NADPH oxidase required for ROS production. RBOHD is activated by Ca^{2+} binding and phosphorylation of conserved residues in its N-terminal (Kadota et al. 2015). Similarly, downstream MAPK cascades are induced however, it is not clear if the upstream activating kinases are the same for PTI and ETI receptors (Lolle et al. 2020).

1.7.6. NLRs function in pathogen recognition.

NLRs can directly recognise pathogen effectors. The rice CNL *Pi-ta* binds the *Magnaporthe oryzae* effector directly *AVR-Pita* (Jia et al. 2000). Flax TNL and M proteins also directly bind with *M. lini* fungal effectors AvrL567 and AvrM (Dodds et al. 2004). Mildew Locus A (*MLA*) directly interacts with its corresponding avirulence effector AVRa and transient coexpression of *MLA/AVRa* in *Nicotiana benthamiana* results in HR triggers cell death. Thus, *MLA* could function in both sensing and signalling (Saur, Bauer, Kracher, et al. 2019). Examples of single

NLR genes that triggered cell death after expression in a heterologous system along with their corresponding *Avr* resulting in a HR response are rust resistance include *Sr35*, *Sr50*, *L6*; *RPP13* (Arabidopsis downy mildew resistance) and *HOPZ-ACTIVATED RESISTANCE1* (*ZAR1*) (Lawrence et al., 2010; Chen *et al.*, 2017; Salcedo *et al.*, 2017; Seto *et al.*, 2017). NLR-mediated disease resistance is effective against obligate or hemi-biotrophic pathogens that cause diseases like rust smut and mildew but not against Necrotrophs like *P. infestans,* and *Z. tritici* that trigger HR in order to feed on dead tissue (Chisholm et al. 2006)

The guard and decoy models endeavour to explain indirect recognition of effectors where either the effector modifies a protein which could be its target (guard model) or its imitator (decoy model) (De Wit et al. 2009; Van Der Hoorn and Kamoun 2008). Recently, some NLRs have been discovered to have non-canonical domains incorporated into their architecture called integrated domains (ID) to enable indirect recognition of pathogen effectors whilst some kinase and DNA-binding IDs seem to be involved in signalling upon effector recognition and help NLRs initiate defence responses (Cesari et al. 2014; Andersen et al. 2020). Several IDs have sequence and structural similarities with effector targets that have been defined including transcription factors or regulators of cell homeostasis (Baggs et al., 2017). This modified target protein is then recognized by the NLR receptor (Win et al. 2012). An early demonstration of the guard model was obtained with the RIN4 protein of Arabidopsis which was shown to form complexes with the NLR proteins RPM1 and RPS2 (Axtell and Staskawicz 2003). Degradation of “guardee” RIN4 by the protease effector AvrRpt2 de-represses RPS2, whilst *Pseudomonas syringae* AvrB or AvrRPM1- phosphorylate RIN4 and activate *RPM1*. Through this, *RPM1* has also been demonstrated to recognize more than one effector (Kim et al. 2005; Mackey et al. 2003). The ID within the NLR interacts directly with effectors upon which immune response is triggered. The *Pst* NLR resistance genes *Yr5*, *Yr7* and *YrSp* encode NLRs zinc-finger BED IDs (Marchal et al. 2018). The resistance of Tobacco mosaic virus by the NLR *N* is also by indirect interaction is the recognition of the p50 effector of the virus and requires NRIP1, a chloroplast-localized rhodanese sulfotransferase. Within the cytoplasm, p50 recruits NRIP1 to the cytoplasm forming a complex that then interacts with *N* (Caplan et al. 2008).

Some NLRs function as pairs where one NLR is involved in sensing the pathogen and is linked to helper/ executor NLR that is requires for signalling to initiate immune response immune signalling (Césari et al. 2014). The rice NLR pair *RGA4/RGA5* recognises the presence of *M. oryzae* avirulence effectors *AVR-Pia* and *AVR1-CO39* via their direct binding to the ID Heavy-Metal Associated domain (HMA or RATX1) which is incorporated within RGA5 after the LRR domain (Cesari et al. 2013b; Okuyama et al. 2011). When AVR-Pia is absent the RGA4/RGA5 pair interacts physically preventing initiation of HR by *RGA4* whilst interaction of the ID with AVR-Pia derepresses RGA4 (Cesari et al. 2014). The products of Arabidopsis *RRS1/RPS4* and rice *RGA5/RGA4* gene pairs form complexes via their N-terminal domains (Williams et al. 2014; Césari et al. 2014). Immune response triggered by detection of *AvrRps4* and *PopP2* by NLR pair *RRS1* and *RPS4* is dependent on the NLRs interacting via their TIR domains (Williams et al. 2014). The that CC domains of Arabidopsis RPW8 can form redundant interaction networks (Wróblewski et al. 2018).

NLRs also form complex networks besides functioning as pairs. Helper NLRs have functional redundancy and are linked with activities of different sensor NLRs (Adachi, et al., 2019). In Solanaceae plants like tomato and tobacco, NLR-required for cell death proteins (NRCs) and NRC-dependent NLRs are part of a complex genetic network conferring resistance to pathogens and pests (Wu et al. 2017). The network evolved from a pair of NLRs that expanded to make up half of the NLRs in some species (Wu et al. 2017). In Arabidopsis, paralogs of the *ADR1* (*ACTIVATED DISEASE RESISTANCE PROTEIN 1*) helper NLRs are essential for the function of many sensor NLRs unknown (Bonardi et al. 2011). Within the genome NRCs are not always clustered with sensor NLRs as in the case of NLR pairs despite being evolutionarily related (Wu et al. 2017).

1.7.7. Engineering NLRs to accelerate development of disease resistant cultivars

The knowledge of NLR structure has enabled the cloning, interspecies transfer, engineering of NLR specificities in the bid to fight crop pathogens and studies of their diversity and evolution (Wulff and Moscou 2014; De La Concepcion et al. 2019; Steuernagel et al. 2015; Witek et al. 2016; Tamborski and Krasileva 2020). The first rust NLR gene to be cloned was *L6* from flax (Ellis et al. 2014). Several other rust resistance NLRs have been cloned from other

crops like maize, soyabeans and barley in addition to those from wheat and its wild relatives. These cereal rust resistance genes were listed in Table 1.5. Cloned genes present the opportunities to introduce multiple resistance genes into a cultivar at a single locus to confer durable resistance. This was accomplished in the case of *Pgt* resistance where five genes were five genes were transferred into a cultivar thus conferring complete resistance (2Blades Foundation 2019). In NLR gene stacking, it is important to combine genes that guard different Avr targets to establish durable resistance. Conversely, NLRs that are susceptibility factors could potentially be edited to render them insensitive to being triggered by pathogens. This was so in the development of resistance to powdery mildew in tomato through the use of CRISPR/Cas9 in generating loss of function of the susceptibility factor SlMlo1 which is an Mlo homologue (Nekrasov et al. 2017). Chemical and irradiation mutagenesis of the NLR Tsn1 in wheat led to insensitivity to ToxA, an effector secreted by *Stagonospora nodorum* and *Pyrenophora tritici-repentis* (Faris et al. 2010).

In addition, the study of IDs could enable discovery of host hubs that are targeted by pathogen effectors (Kroj et al. 2016). It is likely that unintegrated paralogs of NLR IDs could be susceptibility factors that could be edited (Białas *et al.*, 2017). Integrated domains can also be mutated to enhance resistance. De La Concepcion *et al.* (2019) mutated an effector-binding site of the HMA ID of NLR Pikp and this resulted in the expanded binding affinity for *M. oryzae AVR-Pik* alleles that were not recognized by Pikp.

These studies highlight the need to identify the pathogen effectors interacting with NLRs and IDs. Effector identification has lagged behind NLRs which have defined structures and multiple tools that can be used to rapidly identify them from whole genome or sequence capture data. This is particularly so for rust effectors.

1.7.8. NLR evolution.

The evolution of ancestral genes encoding NB domains to present plant NLRs could have been via bacterial intermediary genes, with TIR and NB or TIR and LRR, and may have a common origin with cell-surface LRR receptors (Shao et al. 2014b; Andolfo et al. 2019). Genomic analyses across species show that NB, LRR, and TIR domain-carrying proteins are common

and their structure and makeup have changed over time with gene fusion and fission generating genes with varying functions and stabilities (Andolfo et al. 2019). Transposable elements play a significant role in evolution though facilitating gene duplication or deletion, changing expression or function, and fusing genes from different parts of the genome (Krasileva 2019).

NLR genes are often found in gene clusters and are under strong diversifying selection with the number of NLRs varying between plant species from 54 in papaya, 129 in maize, 245 in sorghum, to 508 in rice (Li et al. 2010). In wheat there are over 3,400 full-length NLR genes that are found mostly on the telomeric regions of chromosomes (Steuernagel et al. 2020). these, of which 1,560 were confirmed as expressed genes with intact open reading frames. However, Andersen et al. (2020) reported just over 2,000 NLR genes in over 500 gene clusters. The differences in number of NLR identified in the different studies was probably due to the tools used in NLR identification with NLR parser being more efficient. Gene clusters are a result of unequal chromosome crossing over (Marone et al. 2013). In eukaryotes, Meiotic recombination rates are at their lowest across centromeres and increase towards the sub-telomeric regions of chromosomes (Baird 2018). Woody plants have higher frequencies of NLRs possibly due to their longer lifecycles which expose them more to pathogens (Yang *et al.*, 2008; Baggs et al., 2017).

NLR expansion can result in new resistance specificities in different ways. Alleles *RPP1* and *L5/L6/L7*, recognise Avr effector alleles (Dodds et al. 2004). Contrary to this, rice and barley homologous NLRs which reside the same clusters detect unrelated rice blast and powdery mildew effectors (Lu et al. 2016). Tomato NLR tomato *Sw-5b* and *I2* recognise viral and fungal pathogens respectively whilst their orthologs in potato recognise oomycete effectors. Whilst their overall homology is high, slight changes in the LRR regions involved in perception causes significant changes in effector specificity (Kourelis and van der Hoorn 2018). Besides widening effector specificities, NLR expansion can have deleterious consequences. This was shown In the model plant Arabidopsis where a single truncated NLR without part of the LRR region, *DM10* (*DANGEROUS MIX 10*), is responsible for hybrid necrosis when it interacts with *DM11* where progeny do not grow past the cotyledon emergence, at three weeks post germination (Barragan et al. 2020). *DM10* arose after *A. thaliana speciation* through interchromosomal

transposition from the *RLM1* cluster, which confers resistance to *Leptosphaeria maculans* (Staal et al. 2006; Guo et al. 2011). Many NLRs carry a fitness cost when there are no pathogens present as a result of consumption of energy through NLR synthesis, regulation or autoactivation (Tamborski and Krasileva 2020).

The advances made so far in the discovery of immune receptors and understanding how they function has potential to impact the development of cultivars with durable resistance to *Pgt* and other pathogens. The deployment of these cultivars requires care to minimise the evolution of pathogen virulence and hence it is imperative to understand pathogen virulence mechanisms. The identification of avirulence effectors can enhance deployment of cultivars with functional *R* gene cassettes. In situations where the Avrs are cloned before their corresponding *R* genes, these can be used to screen for breeding material carrying functional receptors to aid *R* gene cloning. The engineering of *R* genes to expand their effector specificities and knockout of susceptibility factors are certainly exciting tools to complement the search for new resistance receptors from cultivars and crop wild relatives. However, the adoption of these technologies on a large scale is still to be seen due to a largely negative regulatory environment regarding the use of genetic modification and gene editing in resistance breeding despite their economic, health and food security benefits.

1.8. The roles of effectors in plant-microbe interactions

Various organisms such as fungi, oomycetes, bacteria and insects secrete molecules into host plant cells to facilitate successful parasitism or symbiosis through suppression of immunity and modification of physiological processes (Chisholm et al. 2006; Kamoun 2006). The term effector gained widescale use with the discovery that gram-negative bacterial pathogens of plants and animals secreted proteins into host cells via the T3SS (Büttner 2016). This secretion system was first demonstrated for the plant pathogen *P. syringae* and shortly afterwards in the animal pathogen *Yersinia pseudotuberculosis* (Lindgren, Peet, and Panopoulos 1986; Forsberg et al. 1987). Similar to the bacterial T3SS machinery, nematodes and aphids use stylets to inject effectors into plant cells (Mugford et al. 2016). Initially, effectors were identified due to their capacity to trigger HR and were therefore termed Avrs. However, it soon became clear that these proteins had virulence functions in plants that did not carry any corresponding R genes. Also, other pathogen and insect effectors with no known

corresponding R genes have been discovered to have virulence functions. Therefore, the term effector became useful to encompass both virulence and avirulence functions (Hogenhout et al. 2009). Effector genes demonstrate the idea of "extended phenotype" as they are expressed in the pathogen whilst the effect of their products is seen in the host (Dawkins 1999).

The initial discoveries of effectors and the biochemical activities of some of them within the host cell, led to their definition as: "pathogen proteins and small molecules that alter host-cell structure and function" (Hogenhout et al. 2009). Over time, it has been shown that pathogens also secrete molecules such as RNA, DNA, growth regulator analogues, and secondary metabolites which act as effectors to enable successful parasitism. These will be described in detail in the following sections under this topic of effectors. As such the working definition for effectors could be pathogen molecules that are secreted into the plant cytoplasm or apoplast to aid infection. This broad definition would encompass the different types of molecules that are in different pathogen's arsenals in using plants to complete their lifecycles.

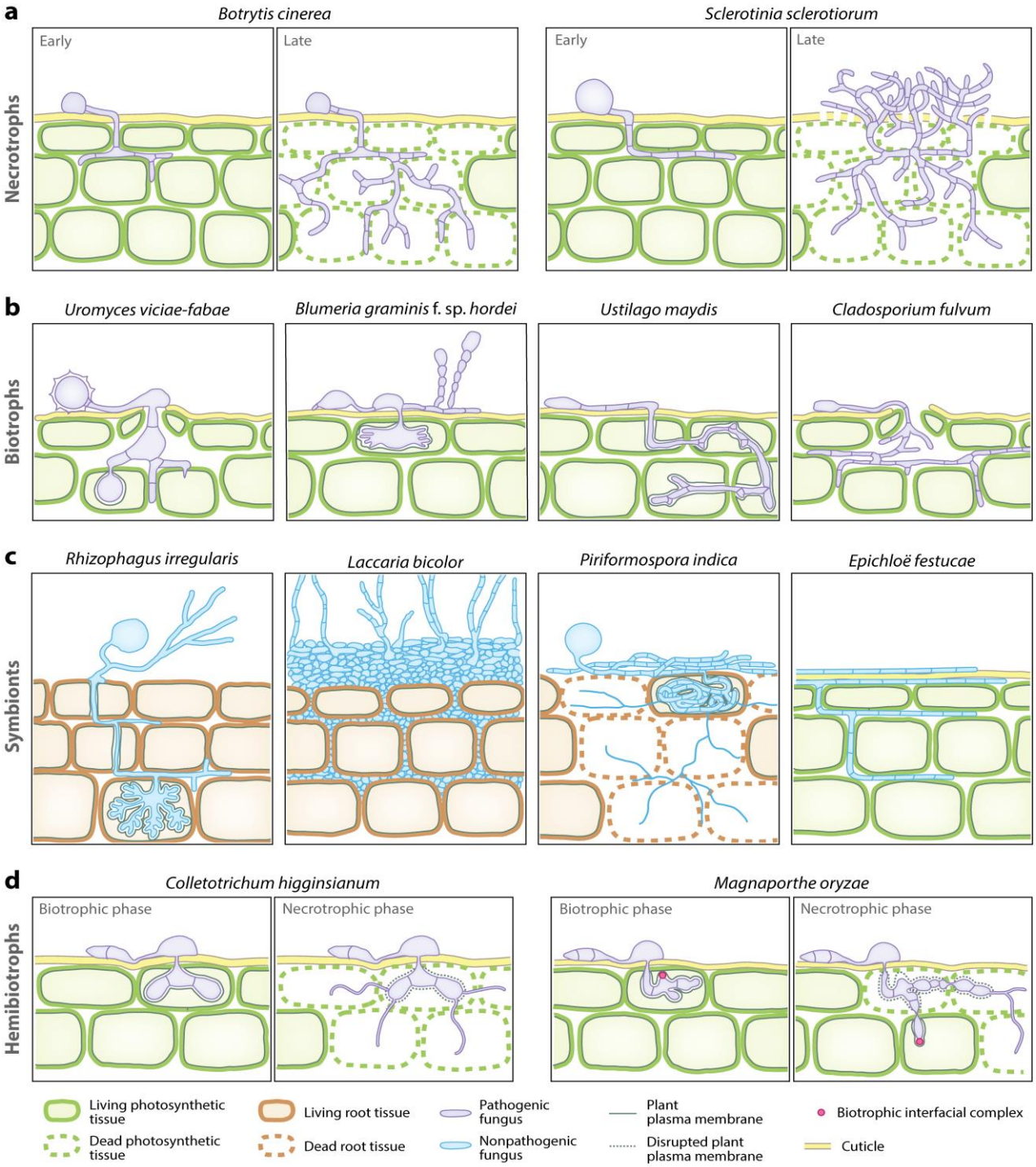
The advent of genomics enabled the discovery of 57 effector families in 19 different strains of *P. syringae* with the strain DC3000 carrying 29 effectors whilst *R. solanacearum* strains contain between 60 to 75 effectors (Peeters et al. 2013; Baltrus et al. 2011). The number of effectors in fungi and oomycetes are much higher. The oomycete *Phytophtora betacei* is closely related to the well-studied *P. infestans* and its predicted secretome has nearly 2,000 predicted effectors, constituting 13% of its proteome (Rojas-Estevez et al. 2020). Of these, 1126 are predicted to localise in the apoplast whilst 807 are translocated to the cytoplasm (Rojas-Estevez et al. 2020). For pathogenic fungi, the genomes of the biotrophic *B. graminis*, *U. maydis*, necrotroph *F. graminearum* and symbiont *Laccaria bicolor* contain 442, 426, 1,049, and 1,161 predicted effectors respectively (Martin et al. 2008). These account for between 5.2 % and 7.5% of the total proteome (Martin et al. 2008). Comparison of the *Pgt* and *Pst* genomes predicted 816 effectors out of 1,857 secreted proteins (43.9%) and 513 effectors from 1,425 secreted proteins (36.0 %) respectively (Xia et al. 2017; Cantu et al. 2013). The secretomes of *Pgt* and *Pst* represent 11.62 % and 9.08% of the total proteome respectively (Xia et al. 2017). These fungal candidate effectors were selected based on classical criteria

Saunders et al., 2012 and Cantu et al. (2013) where small proteins (<300 amino acids), with N-terminal secretion peptides, no homology to known proteins, no known domains, absence of transmembrane domains, presence of a nuclear localisation signal, internal repeats and cysteine residues. However, far fewer effector proteins have been verified.

One contributing factor to the large number of oomycete and fungal effectors are gene duplications which result in functional redundancy. Network analysis of plant-pathogen interactions has shown that multiple effectors converge on key plant molecules linked to multiple pathways (nodes) (Ahmed et al. 2018). This explains why experiments to determine the virulence function of identified effectors have largely not been successful (Saitoh et al. 2012). Disruption of 78 effector genes that are highly expressed during early infection of rice colonization by *M. oryzae* determined just one gene required for virulence. Also, deleting multiple (six) *mig2* genes had no impact on *U. maydis* tumour development upon infecting maize plants (Farfsing et al. 2005).

1.9. Fungi and oomycetes secrete effectors that localise to the apoplast or cytoplasm.

Effectors can be classed into two broad groups according to the sites that they target within the host plant. Apoplastic effectors are secreted into the plant apoplast. The apoplast is made up of a continuum of cell walls of neighbouring cells and extracellular spaces (Münch 1930; Sattelmacher 2001). The apoplast facilitates the rapid transport of water and solutes and has an acidic pH (Sattelmacher 2001). Apoplastic effectors are secreted into this environment where they interact with extracellular target PRRs, proteases and hydrolases (Tian et al. 2007). These effectors are typically cysteine-rich which allows for stabilisation of their tertiary structures and association by disulfide bridges thus, they have evolved to survive the conditions in the plant apoplast (Stergiopoulos et al. 2013). On the other hand, cytoplasmic effectors function within host cells and have a diverse range of targets and activities such as suppression of PTI/ ETI, modification of plant metabolism, acquisition of nutrients and inducing necrosis. Cytoplasmic effectors are translocated inside the plant cytoplasm via structures like the haustoria and intracellular hyphae, or other membranous structures (**Fig 1.3**; Lo Presti et al. 2015).



Reproduced from (Lo Presti et al. 2015b).

Figure 1.5 | Infection structures produced by fungi with different lifestyles

(a) Necrotrophic fungi like *Botrytis cinerea* and *Sclerotinia sclerotiorum* grow in the apoplast just below the epidermis. They secrete toxins to kill the epidermal cells. (b) Obligate biotrophs such as rusts and mildews, e.g., *U. viciae-fabae* and *Blumeria graminis* f. sp. *hordei* (*Bgh*), form haustoria within the host cells. The smut fungus *U. maydis* initially grows in the intracellular space and then reverts to intercellular growth. The biotrophic tomato pathogen *C. fulvum* colonizes the apolast. (c) The obligate mycorrhizal symbiont *Rhizophagus irregularis* colonizes

single root cortical cells with branched arbuscules. *L. bicolor*, an ectomycorrhizal fungus, grows exclusively intercellularly; colonizes roots by forming a mantle or sheath of hyphae, which grows between cortical cells. (d) Hemibiotrophic fungi like *Colletotrichum* spp. and *M. oryzae* first develop bulged biotrophic infection hyphae that later change into thin necrotrophic hyphae. Intracellular structures are produced and are surrounded by the plant cell membrane and the membranous biotrophic interfacial complex structure in *M. oryzae*–infected cells are shown in pink.

Necrotrophic fungi do not enter plant cells and grow within the apoplast where they secrete effectors and toxins to trigger cell death (Fig. 1.5a). Biotrophic and hemibiotrophic fungi and oomycete pathogens require living host cells (Fig. 1.5b). These secrete effectors into the apoplast or cytoplasm via structures such as appressoria or haustoria (Kemen et al. 2005; Catanzariti et al. 2006; Rafiqi et al. 2010; Koeck, Hardham, and Dodds 2011). Upon entering the cell walls of the epidermis, lobed haustoria develop within the mesophyll (Manners and Gay 1983). The corn smut fungus and biotroph, *U. maydis* does not form haustoria. Instead, its intracellular hyphae are enveloped by the plant cell plasma membrane to create a bi-directional interface for exchange of molecules (Djamei and Kahmann 2012). Hemibiotrophic pathogens like *M. oryzae* and *Z. tritici* grow as biotrophs during early infection stages and switch to a necrotrophic phase by killing their host cells (Fig. 1.5d). *M. oryzae*, utilises two secretion systems for apoplastic and cytoplasmic effectors (Giraldo et al. 2013). Effectors are secreted into the cytoplasm via the biotrophic interface complex at the tip of the initial bulbous cell. Apoplastic effectors are delivered via intercellular hyphae (Zhang and Xu 2014). It is not yet known how *L. maculans*, a hemibiotrophic pathogen that causes black leg disease in *Brassicas* plant species, delivers effectors (Selin et al. 2016).

1.10. Known bacterial, fungal and oomycete effector characteristics.

Many cloned effectors and *Avr* effector genes in fungi and oomycetes encode small, secreted proteins. Their amino acid (aa) lengths range from 28 for the effector protein Avr9 of *C. fulvum* to 314 aa for the RXLR effector AvrM from *M. lini* (De Wit et al. 1997; Selin et al. 2016). However, a few effectors are much larger than this. *Pgt* effectors longer than 314 amino acids include *AvrSr35* (578 aa), apoplastic effectors RGDBP with an RGD motif (818 aa) and VPS9 (744 aa) (Nirmala et al. 2011; Salcedo et al. 2017). *M. oryzae* Avirulence Conferring Enzyme1

(ACE1) is recognized by the resistance gene Pi33 (Böhnert et al. 2004). ACE1 is the largest known effector with 4,035 aa (Böhnert et al. 2004).

Protein effectors in eukaryotic pathogens are secreted via the endoplasmic reticulum-Golgi apparatus and exocytosis. This requires an N-terminal secretion signal for translocation (Ellis et al. 2009; Catanzariti et al. 2006; Whisson et al. 2007). There are exceptions to this where no clear consensus signature is identified that is linked with translocation and uptake into the plant cell (De Wit et al. 2009; Stergiopoulos and De Wit 2009). Effectors known to function in the cytoplasm like *Bgh* Avr10 and Avrk1, do not have a secretion signal peptide (Ridout et al. 2006a). The *Pgt* AvrSr35 has a secretion peptide and is recognised within the cytoplasm by the NLR Sr35 (Salcedo et al. 2017). Infiltration of the purified effector into the leaves of wheat carrying Sr35 results in HR suggesting that the effector is translocated into the cytoplasm from the apoplast by some unknown mechanism (Salcedo et al. 2017).

Some effectors do have identifying features. A well-studied translocation motif, RXLR-dEER is located near to the N-terminus of many oomycete effectors (Morgan and Kamoun 2007; Whisson et al. 2007). Effector predictions based on this motif from *Phytophthora* genomes have unveiled several RXLR effector genes in *P. infestans* (500), *P. ramorum* (374) and from *P. sojae* (396) (Jiang et al. 2008a; Haas et al. 2009). *P. betaci* has the largest number (791) of RXLR effectors known so far (Rojas-Estevez et al. 2020). Some variations of the RXLR motif are KXLR, RXLG and PXLR (Jiang et al. 2008a). Several RXLR effectors can suppress immunity (Whisson et al. 2016). Of these, AVR3a from *P. infestans* which is recognised by R3a is well studied (Armstrong et al. 2005). Avr3b from *P. sojae* is a Nudix hydrolase secreted into plant cells to impair host immunity (Dong et al. 2011). The *Hyaloperonospora arabidopsidis* CYSTEINE-RICH PROTEIN (HaCR1), which suppresses immunity and also acts as a protease inhibitor in the apoplast, does not have an RXLR domain (Dunker et al. 2021).

Oomycetes, secreted effectors also share LxLFLAK motifs (Phe, Leu, Ala, Lys) or CRN (Crinkling and Necrosis inducing effector motifs) (Jiang et al. 2008a). PiCRN8 is 599 aa long and is localised in the cytoplasm where it triggers plant cell death. It also carries a C-terminal kinase-like domain that could play a role in modification of host cell signalling (Schornack et al. 2010). CRN functions and role in virulence are mostly unknown.

The *Pst* gene *Ps87* encodes a protein with high similarity to *M. lini* candidate effector protein 767 and can enter plant cells via an RXLR-like motif, [K/R]RLTG (Zhang et al. 2006; Gu et al. 2011). This motif [K/R]RLTG is similar to the RXLR-like motif of the Avr1b (Kale et al. 2010). Ps87 has homologs in other fungi such as *Pgt*, *M. laricis- populina*, *M. oryzae*, *Alternaria brassicicola* and *P. tritici-repenti* (Gu et al. 2011).

The Y/F/WxC motif– where the first amino acid is tyrosine, phenylalanine, or tryptophan, whilst the last is always cysteine–is present in the rust effector AvrL567 of *M. lini* and Avr2 and Avr4 of *C. fulvum.* This motif was used as a characteristic for selection of effectors from the predicted secretomes of *Pgt* and *M. larici-populina* (Saunders et al. 2012). Studies of other genomes have identified Y/F/WxC-protein genes from *Bgh* (107), *Pgt* (178) and *Pt* (57) (Godfrey et al. 2010; S. Zhao et al. 2020).

Several validated effectors of *M. oryzae* are known as the MAX (MagnaportheAVRs and ToxB like) effectors (de Guillen et al. 2015; Białas et al. 2018). As observed with effectors from other pathogens MAX effectors have low sequence similarity but are structurally more similar. MAX effectors, have a conserved fold made of six β-sheets which are arranged in alternating directions (Zhang et al. 2013). This effector is largely expanded in *Magnaporthe* and makes up to 10% of the predicted effectors and 50% of verified effectors (de Guillen et al. 2015). Effector motifs enable better effector prediction from genome sequences compared to sequence similarity analyses (Franceschetti et al. 2017).

Additional genomic features, such as effector gene clusters separated by long intergenic regions, internal repeats and no protein family domains with exception of those with known pathogenicity have been used in a comprehensive hierarchical clustering method to screen for effectors in rusts (Haas et al. 2009; Raffaele et al. 2010; Saunders et al. 2012).

Several *Avr* genes have been identified in *B. graminis*: *AvrPm3*$^{a2/f2}$ and *AvrPm2* in *Bgt*; *Avra1* and *Avra13* in *Bgh*. *SvrPm3*$^{a1/f1}$ suppresses recognition of one of these *Avrs* proteins in wheat (Bourras et al. 2015; Spanu 2017; Praz et al. 2017; Lu et al. 2016). Of these *Avr* genes, four encode proteins with features resembling inactive RNases. They are part of a superfamily of RNase-Like Proteins associated with Haustoria (RALPH) effectors. This superfamily was

examined in the *Bgh* genome sequence where 491 candidate effector genes were predicted of which 54 encode RALPH proteins (Pedersen et al. 2012). Silencing of two *Bgh* RALPH effector genes BEC1011 and BEC1054 hampers virulence (Pliego et al. 2013).

The biotrophic fungal pathogen *U. maydis* secrets Pep1 that inhibits maize POX12, which is a peroxidase and is conserved in the ROS production pathway (Hemetsberger et al. 2012). Mutants of *pep1* induce defence responses and show reduced penetration of epidermal cells and hampered growth of intracellular hyphae (Doehlemann et al. 2009). The vascular wilt fungus *F. oxysporum* f. sp. *lycopersici* (*Fol*) utilises 14 effector proteins that are secreted into the xylem termed Six (secreted-in-xylem) proteins (Takken and Rep 2010; Schmidt et al. 2013). *Six4/Avr1* is the first Avr gene to be cloned (Jones 1988). *Six4* suppresses the ability of R proteins I-2 and I-3 to confer resistance against Fol race 1, even though isolates within this race group secrete their corresponding Avr2 and Avr3 (Houterman et al. 2008). *Avr2/Six3*, *Avr3/Six1*, and *Six6* are known to function in virulence in susceptible tomato lines (Takken and Rep 2010). Coexpression of AVR2/Six3 without its signal peptide and I-2 in *N. benthamiana* results in HR, thus Avr2 is recognized inside the cell. Six6 inhibits Avr2-induced ETI (Takken and Rep 2010). *Six* genes have also been identified in *L. maculans* (S*ix1* homologue) whilst *Six1* and *Six6* homologues are present in *C. orbiculare* and *C. higginsianum* (Kleemann et al. 2012; Gan et al. 2013; Van de Wouw et al. 2010).

Bacterial effectors such as *P. syringae* effectors AvrPto, AvrRpt2 and AvrRpm1 act to inhibit PTI (Hauck et al. 2003; Kim et al. 2005). In addition, *X. campestris* effectors suppress PTI and bacteria lacking the T3SS cannot evade host detection of their PAMPs (Keshavarzi et al. 2004). Some effectors like AvrRpt2 utilise plant metabolic pathways to enable function. This effector from the *P. syringae* is inactive when it is secreted into the cytoplasm where it is then activated by eukaryotic cyclophilins (Coaker et al. 2005). Once active, AvrRpt2 suppresses PR (Pathogenesis Related) genes *PR-1*, *PR-2* and *PR-5* expression during infection. AvrRpt2 also suppresses SA production thus promoting virulence.

1.11. Effectors with enzymatic activity.

Despite the broad criterion of effectors not having a known function, some microbial effector proteins do have enzymatic activity. *U. maydis* secretes a chorismate mutase Cmu1 which functions as a virulence factor (Djamei et al. 2011). Upon secretion into host cells, this enzyme can move to other cells and alters their metabolic status. Chorismate mutases are key enzymes in the shikimate pathway which produces aromatic amino acids such as tyrosine and phenylalanine (Tohge et al. 2013). The soybean cyst nematode *Heterodera glycines* also secretes chorismate mutase to affect plant cell development (Bekal et al., 2003). *F. graminearum* effectors LIP1, a lipolytic enzyme and XYLA, an endo-1,4-β-xylanase have been shown to elicit immune reactions in plants (Sella et al. 2013; Feng et al. 2005). The effect of XYLA in inducing PTI has been observed for other fungi like *Trichoderma* and *B. cinerea* (Sella et al. 2013). LIP1 is a 598 amino acid-long effector that is secreted for lipid hydrolysis and is necessary for fungal growth (Feng et al. 2005). Secreted lipases are present in other fungi such as *B. cinerea* and yeasts (Feng et al. 2005). A study of *M. lini* proteins secreted from haustoria identified effector AvrP123 that has homology to a Kazal-serine protease inhibitor (Catanzariti et al. 2006).

1.11.1. Effectors that target hormone regulated defence and hormone production.

Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are key hormones that regulate plant defence against pathogens (Kazan and Lyons 2014). ET acts in response to pathogens and herbivores (Shen, et al 2018). SA-dependent immunity defends against biotrophic pathogens whereas JA is involved in defence against Necrotrophs (Kunkel and Brooks 2002). SA and JA defence pathways counteract each other and thus pathogens exploit this (Kunkel and Brooks, 2002). *Pseudomonas* bacteria produce coronatine that mimics JA and suppresses SA-initiated defences (He et al. 2004; Reymond and Farmer 1998). Several effectors manipulate JA synthesis working together including AvrB, AvrRpt2, AvrPphB, HopPtoK, and AvrPphEpto (He et al. 2004).

To manipulate plant hormone production, *Agrobacterium tumefaciens* transfers a part of its tumour-inducing plasmid carrying multiple virulence (*vir*) genes which become integrated into the host cells genome (Chilton et al. 1980). These *vir* genes are highly expressed and

encode enzymes of auxin and cytokinin, which results in cell proliferation and expansion causing crown galls to develop. Other *vir* genes are involved in opine synthesis which provides a food source for the bacteria (Nester 2015; Lee et al. 2009). Elevated auxin levels in Arabidopsis infected by *Agrobacterium* modulated ET and SA production and suppressed the initiation of defence (Lee et al. 2009).

The symbiotic mycorrhizal fungus *Rhizophagus irregularis* transmits signals to reprogramme gibberellic acid synthesis in *Medicago truncatula* causing expansion of the root cortex to facilitate arbuscule development (Heck et al. 2016). Mycorrhiza induced Small Secreted Protein 7 (MiSSP7) from *L. bicolor* blocks JA signalling in *Populus trichocarpa* allowing for colonization (Plett et al. 2014). *M. oryzae*, which has a hemibiotrophic lifestyle, produces and secretes a hydroxylated JA derivative (12OH-JA) as a metabolite effector (Patkar et al. 2015). The effector suppresses JA signalling whilst promoting flower and tuber growth immunity during the biotrophic phase (Patkar et al. 2015). A secreted chorismite mutase from *U. maydis* reduces synthesis of SA to enable growth of the biotrophic fungus (Djamei et al. 2011). The necrotrophic *Cochliobolus miyabeanus*, produces large amounts of ET via its ethylene-forming enzyme resulting in ET-signalling in rice (Van Bockhaven et al. 2015).

1.11.2. Elimination of competition: anti-microbial activity.

The effector VdAve1, secreted by *V. dahliae*, exhibits antimicrobial activity (Snelders et al. 2020). VdAve1 manipulates the microbiomes of cotton and tomato by suppressing antagonistic bacteria to facilitate infection. This small, secreted cysteine-rich effector protein is recognised by its corresponding R protein Ve1 (De Jonge et al. 2012). *VdAve1* is a virulence effector on plants that lack *Ve1* (De Jonge et al. 2012). VdAve1 has homology with plant natriuretic peptides (PNPs) that have been identified in numerous plant species and are involved in biotic stress response and homeostasis of ions and water (Ficarra et al. 2018). However, the mode of action of VdAve1 to contribute to fungal virulence is still to be determined.

1.11.3. Evading/ suppressing defences.

Pst effector Pst_8713 could suppress HR triggered by the mouse apoptotic protein-BAX or *P. infestans* PAMP INF1 (Zhao et al. 2018). ETI triggered by an avirulent *Pst* isolate was reduced

when *Pst_8713* was overexpressed in wheat leaves. Knockdown of *Pst_8713* by HIGS resulted in production fewer uredinia (Zhao et al. 2018). Overexpression of effector *Pstha5a23* in *N. benthamiana* was also found to suppress HR. However, its silencing did not affect virulence (Cheng et al. 2017).

The soybean root rot pathogen *P. sojae* secretes an RXLR effector, Avr3b which also has a Nudix hydrolase motif (Dong et al. 2011). When expressed in *N. benthamiana Avr3b* increased susceptibility to *P. capsici* and *P. parasitica*, and decreased ROS accumulation. Dong et al., (2011) also determined Avr3b to be an ADP-ribose/NADH pyrophosphorylase, as predicted from the Nudix motif and subsequently confirmed this biochemically where removal of the Nudix motif abolished enzyme activity. There are Nudix hydrolases that are negative regulators of plant immunity (Ge et al. 2007). Thus, the presence of this motif in other *Phytophthora* species could indicate that this might be how they suppress plant immunity (Dong et al. 2011).

The effector Ecp6 of *C. fulvum* effector has a LysM domain, which is homologous to 16 putative *effector* proteins from: *Aspergillus niger, M. orzae, M. fijiensis, Z. tritici, B. cinerea, S. sclerotiorum, A. nidulans, A. oryzae, A flavus, C. lindemuthianum*, and *L. maculans* (Bolton et al. 2008). LysM domain-carrying effectors are cysteine-rich and are secreted in the apoplast by many fungal pathogens to act as decoys that prevent PTI in some cases by tightly binding to chitin oligomers and thus preventing recognition of the PAMP chitin recognition by PRRs (Marshall et al. 2011; Sánchez-Vallet et al. 2020).

1.11.4. RNA effectors in plant-fungus/oomycete interactions.

As detailed in section 1.6.4 on the application of RNAi in the control of plant diseases, emerging evidence shows that small RNAs (sRNAs) are involved in unrelated species–known as cross-kingdom talk/ trans-kingdom RNAi–moving between plants and pathogens to silence gene expression (Weiberg et al. 2013; Kuan et al. 2016; Wang et al. 2016). This was initially in *B. cinerea* which transfers siRNAs to *Arabidopsis* suppressing the expression of host immunity genes**.** Upon infection, several genes were targeted by different sRNAs: mitogen-activated protein kinase 2 (MPK2) and MPK1, were targeted by sRNA Bc-siR3.2; the sRNA Bc-siR3.1 silenced peroxiredoxin (oxidative stress response gene), and a WAK

which is suppressed by Bc-siR5 (Weiberg et al. 2013). These sRNAs originate from LTR retrotransposons and two Dicer-like enzymes of the fungus are involved in their production (Weiberg et al. 2013). In the cell, they bind to plant Argonaute (AGO) proteins to silence RNAi genes involved in immunity. Mutant *Arabidopsis* AGO1 mutants are resistant to *V. dahlia* and its sRNAs have predicted targets suggesting that the verticillium wilt pathogen employs RNAi to suppress immunity during infection (Ellendorff et al. 2009; Wang et al. 2016). However, how they are delivered into the host cells is still unknown.

Recently, effectors that suppress defence-related RNA silencing suppression have been discovered in *Phytophthora* (Xiong et al. 2014; Hou et al. 2019). *Phytophthora* infection of Arabidopsis leads to increased production of a diverse pool of secondary small interfering RNAs (siRNAs). *P. capsici* effector PSR2 blocks HIGS through suppression of the production of antimicrobial siRNAs (Hou et al. 2019). These siRNAs are delivered by plants in extracellular vesicles probably to silence pathogen genes during natural infection. When these plant siRNAs are introduced in *Phytophthora*, development is impaired and eliminates virulence (Hou et al. 2019). As described earlier in this review, plants also employ RNAi to silence pathogen genes. An example is the miRNA PC-7484 from wheat which targets transcripts of Ubiquilin, which is a ubiquitin-like protein involved in protein degradation in *Pst* (H. Feng et al. 2016).

1.11.5. Effectors that have a structural role.

The rust effector Uf-RTP1p, is conserved in *U. viciae-fabae* and *U. striatus* that is secreted via the haustorium and carries a putative nuclear localisation signal (Kemen et al. 2005). It operates within the plant cell to form fibril structures whose function is to stabilize the colonized cell (Kemen et al. 2013).

1.11.6. Toxin effectors.

Necrotrophic pathogens induce cell death using secreted necrotrophic effectors (NEs) that interact with dominant susceptibility genes in an “inverse gene-for-gene” relationship thus activating cell death and defence responses in favour of the pathogen (Winterberg et al. 2014; Shi et al. 2016). *Cochliobolus victoriae*–the causal agent of victoria blight of oats–delivers a chlorinated cyclic pentapeptide, victorin (Wolpert et al. 1988). The induction of cell death by

this toxin is dependent on the NLR LOV1 (Wolpert et al. 1988). Snn1, is a Wall-Associated Kinase protein in wheat that confers resistance to biotrophic pathogens. However, Snn1 enhances susceptibility to strains of *Phaeosphaeria nodorum* that secretes Tox1, which results in cell death upon its recognition (Shi et al. 2016). About nine host susceptibility gene/NE pairs have been characterized in this crop-pathogen interaction. The *Tsn1*-SnToxA, *Snn1*-SnTox1, and *Snn3-B1*-SnTox3 interactions have been studied more intensively due to the cloning of either the susceptibility gene or the pathogen toxin effector protein and are reviewed by Haugrud et al., (2019). The *ToxA* gene is particularly important as it is found in *P. tritici-repentis* and was likely acquired by *Bipolaris sorokniana*, *Colchliobolus heterostrophus* and *P. nodorum* by horizontal transfer and became an important virulence factor (Friesen et al. 2006; Friesen et al. 2018; McDonald et al. 2018; Lu et al. 2015).

1.11.7. Secondary metabolites as effectors.

Secondary metabolites can function as non-proteinaceous effectors that are host or nonhost-specific toxins and as Avrs, defence suppressors and hardeners of fungal cell walls (Collemare et al. 2019). The understanding of the biological function of fungal SMs was limited to those that are produced *in vitro* or detectable in plants. However, the application of transcriptomics has revealed numerous fungal secondary metabolite biosynthesis gene clusters that are active during infection. Half of the clusters in *M. oryzae* are highly expressed in the biotrophic phase in rice and barley (Collemare et al. 2008; Collemare et al. Lebrun 2019). *Z. tritici* expresses 11 biosynthetic gene clusters during its asymptomatic stage in wheat and at the start of the necrotrophic phase (Rudd et al. 2015). The secondary metabolite toxins trichothecenes, are a major class of mycotoxins produced by *Fusarium*, are toxic to both plants and mammals (Cutler 1988). These include type A and type B trichothecenes like the T-2 toxin and deoxynivalenol. *Fusarium* trichothecenes cause necrosis, chlorosis, and mortality by disrupting protein synthesis enabling the development of many plant diseases such as wilts, stalk rot, root rot and leaf rot in economically important crops (Cheeke 1998; Wang et al. 2006). Trichothecenes from *Myrothecium*, and *Stachybotrys* cause sick building syndrome as a result of the presence of their spores contaminating indoor air (Kuhn and Ghannoum 2003).

M. oryzae secretes Indole Acetic Acid (IAA) in its biotrophic phase around the infection hyphae although it is unknown how this benefits the pathogen (Tanaka et al. 2011). *U. maydis* produces IAA to induce tumour infection at infection sites (Reineke et al. 2008).

1.12. Effector evolution.

Effector variation is driven by genomic modifications that range from point mutation, sexual recombination, chromosomal structural variation, horizontal gene transfer and somatic hybridisation that affect gene composition in genomes (Raffaele and Kamoun 2012; Park and Wellings 2012; Dong et al. 2015; Chen et al. 2017; Li et al. 2019). The evolution of effector content in rusts by sexual recombination and somatic hybridisation was covered earlier in this introduction. TEs like Miniature Inverted-repeat Transposable Elements (MITEs) promote genomic re-organization and environmental adaptation to the environment especially to new host resistance in the “arms race” as highlighted previously for *Six* genes in Fusarium and *Pgt* (Schmidt et al. 2013; Salcedo et al. 2017). Several pathogens have a genomic arrangement that follows the so-called "two-speed genome" model and regions with effectors that have a high content of TEs and repetitive sequences that are regarded as ensuring rapid adaptive evolution whilst separate from stable regions with housekeeping genes (Dong et al. 2015).

However, rust pathogen genomes like *P. graminis* (*Pgt*)*, P. coronata* and *P. striiformis* (*Pst*) do not follow this model and predicted secreted proteins are located in regions with genes and not repetitive elements (Schwessinger et al. 2018; Miller et al. 2018; Li et al. 2019). A *Pgt AvrSr35* allele had a 400 bp MITE insertion in one of its exons which caused a premature stop codon was identified in the American race RKQQC and TTKSK (Ug99) that originated in East Africa (Salcedo et al. 2017; Li et al. 2019). This suggests that some isolates virulent on *Sr35* resulted from TE insertion resulting in the truncation of AvrSr35 (Salcedo et al. 2017). In the *Fol* genome, effector genes are present in regions rich in TEs (Schmidt et al. 2013). The search specifically for MITEs allowed for identification and validation of candidate effectors (Schmidt et al. 2013). However, these MITEs were in promotor regions and did not disrupt gene function as in the case of the non-functional *AvrSr35* allele. Repeat Induced Point mutations (RIPs) protect against TEs by generating G:C to A:T mutations that inactivate genes and were first identified in the model fungus *N. crassa* (Kinsey et al. 1994). These occur during crossing, affecting DNA that may carry duplicated non/coding regions (Van de Wouw et al. 2010).

The genomes of rusts and mildews are larger than most pathogens (Duplessis et al. 2011b). They have clear features of obligate biotrophy such as an expansion of predicted effectors and incomplete pathways for important nutrients like nitrogen and sulphur and expanded transporter genes for amino acids (Duplessis et al. 2011b). The pathogens have elevated levels of transcripts for small secreted proteins, hydrolytic enzymes, and transporters within plant tissues suggesting that these function in infection and assimilation of nutrients from the hosts (Duplessis et al. 2011b). The ability of some rust fungi to switch between unrelated hosts could indicate that they might have separate effector repertoires to infect different hosts (Lorrain et al. 2019). Yet, despite bacterial pathogens having lower numbers of effectors, they can have a broad host range. Examples include *P. syringae* and *Xanthomonas* sp. (24 monocot and 268 dicot species). Thus, effector numbers cannot fully account for the requirement for large numbers of effectors by filamentous, macrocyclic pathogens (Leyns et al. 1984; Morris et al. 2019). Different effectors can also be required to infect different tissues on the same host. This was established by a transcriptome study of *U. maydis* where nearly 45% of the genes encoding secreted proteins were expressed exclusively during infection of either floral or vegetative parts (Redkar et al. 2017).

Effector genes in pathogens such as *Bgh*, *L. maculans* and *Z. tritici* are experiencing positive selection (Fouché et al. 2018; Menardo et al. 2017). *Z. tritici AvrStb6* contains 16 non-synonymous SNPs pointing to rapid evolution (Zhong et al. 2017). The gene is in a sub-telomeric region flanked by transposable elements, which could likely facilitate the breakdown of *Stb6* resistance (Zhong et al. 2017). *Avr* genes seem to be under a higher selection pressure than other effector genes. In *M. oryzae*, the evolution of six known *Avrs* and seven non-*Avr* in 62 strains showed frequent presence/absence polymorphisms and 10-fold nucleotide variation compared to non-*Avr* (Huang et al. 2014). *Avrs* also had high d_N/d_S (non-synonymous to synonymous ratios), and shared non-synonymous substitutions plus proximity to repeat sequences (Huang et al. 2014).

Horizontal gene transfer can result in added virulence. *V. dahliae* effector *VdAve1* has homology with plant natriuretic peptides (PNPs). PNPs are present in numerous plant species and are involved in biotic stress response and homeostasis (Ficarra et al. 2018). This suggests

that *VdAve1* could have been assimilated from plants via horizontal gene transfer (De Jonge et al. 2012). The majority of the 11 *Six* effector genes in *Fol* reside on a lineage-specific chromosome needed for successful infection of tomato (Ma et al. 2010). Lineage-specific regions in *Fusarium* sp. are rich in transposable elements and unique genes related to pathogenicity acquired via horizontal transfer (Ma et al. 2010). Experimental transfer of two lineage-specific chromosomes between strains of *F. oxysporum* conferred pathogenicity to a non-pathogenic strain (Ma et al. 2010). Transfer of the *P. tritici-repentis ToxA* gene into avirulent isolates confers virulence (Ciuffetti et al. 1997; Ohm et al. 2012). Fungi that lack *P. tritici-repentis ToxA* are mildly virulent on wheat carrying *Tsn1*, whilst cultivars that lack *Tsn1* are less susceptible. About 24% of *S. nodorum* field isolates carry *ToxA* whilst it is present in 80% of *P. tritici-repentis* isolates (Liu et al. 2004; Friesen et al. 2006). This balance is seen to be a result of selection on cultivars that carry or lack *TnS1* leading to the high incidence of deletion variants in both pathogens (Friesen et al. 2018). In Australian isolates of *Bipolaris sorokiniana* became virulent through the horizontal acquisition of *ToxA* from *P. tritici- repentis* (McDonald et al. 2018).

Altogether, these mechanisms by which effectors are created, gained, or lost by pathogens in the "arms race" with their respective hosts shows how complex their genetics is and the advantages they have on an evolutionary scale. The understanding of how this occurs has begun to take shape for important pathogens like *Phytophthora* sp*., Magnaporthe* sp., *Fusarium* sp., *L. maculans* and *U. maydis* where some studies on effector evolution have replicated mechanisms such as RIP mutations and horizontal gene transfer in the lab. This is not so, however, in the case of rust pathogens as effector cloning and genetic transformations are hampered by their obligate biotrophic lifestyle. Bioinformatic candidate effector identification pipelines have only revealed few candidate *Avr* effectors and so far, these effectors have no distinct effector features like motifs that could lead to the identification of other effectors. Furthermore, wheat transient expression assays are not reliable and thus studies to determine effector targets and function within the host are not yet feasible. Effector validation experiments thus rely on the expression of effectors in plant species that are distinct from the pathogen native host and could lack the cloned effector target molecules.

1.13. Identification of candidate effectors.

The first plant pathogen effector that was identified was from *P. syringae* pv. *glycinea* where a genomic library of the pathogen was constructed in plasmid vectors and maintained in *E. coli* (Staskawicz et al. 1984). Conjugations with a virulent strain of *P. syringae* pv. *glycinea* and screens were conducted for a change to avirulence through screening for the HR phenotype (Staskawicz et al. 1984).

Many fungal effectors were initially identified, using map-based cloning such as *AvrPi-ta*, *ACE1*, and *Avr-CO39* from *M. oryzae*; *AvrLm1*, *Avrlm4-7* and *AvrLm6* from *L. maculans*; *AvrPm3*$^{a2/f2}$ from *B. graminis tritici* (Chauhan et al. 2002; Böhnert et al. 2004; Gout et al. 2006; Fudal et al. 2007; Parlange et al. 2009b; Bourras et al. 2015). More recently, a *Pst* mapping population was generated by selfing and it was determined that virulence was a dominant trait whilst avirulence was recessive (Yuan et al. 2018). This was also established in *Pgt* crosses (Bushnell 1984). Avirulence loci need to be homozygous for the gene to be identified. Some avirulence genes can change from dominant to recessive depending on the isolate (Tian et al. 2016; Yuan et al. 2018). For cereal rusts map based cloning is limited by the difficulty of producing teliospores under controlled conditions and the homozygosity of some *Avr* genes (Yuan et al. 2018).

The advent of whole genome and transcriptome sequencing has improved effector identification. Analysis of a cDNA from barley tissue infected by *Bgh* showed genes encoding small secreted proteins with signal peptides and [YFW]xC motifs (Godfrey et al. 2010). Searching for effectors using known structures enables predictions of putative effectors as in the case of effector genes in *Phytophthora* sp. (RXLR-dEER motif), *L. maculans* (RIP mutations) and powdery mildew (Jiang et al. 2008b; Haas et al. 2009; Rouxel et al. 2011; Tyler et al. 2006). Even though some effectors seem to have common sequence features, effector prediction faces challenges due to use of wide-ranging criteria which are dependent on limited information from the few cloned effectors. The majority of cloned effectors are proteins with a secretion signal, are small in size (<300 aa), lack homology with known proteins and are cysteine-rich (Saunders et al. 2012; Sperschneider et al. 2016b). As described previously in this review not all effectors are proteins, and effector proteins can be larger than 300 amino acids and can have homology with known proteins. The *L. maculans*, *AvrLm1* is a cytoplasmic

effector that has only one cysteine residue (Gout et al. 2007). Thus, searching for cysteine-rich proteins is important mainly for apoplastic effectors.

Effector prediction can require combining different methodologies. In the *Fol* genome, effector genes are present within subregions enriched for DNA transposons (MITEs). The miniature Impala (mimp) TE featured consistently in effector promoters and this became a criterion to identify candidate effector genes (Schmidt et al. 2013). In combination with proteomics of xylem sap samples extracted during infection, 16 predicted effectors were identified in the isolate Fol4287. These included the known *Six2*, *Six3*, *Six6* whereas *Six1* and *Six7* could not be identified due to unassembled regions in the reference assembly for the isolate (Schmidt et al. 2013). This highlights the importance of the quality of the reference genome when predicting effectors. Bioinformatic tools also have limitations. Schmidt et al., (2013) could not identify the positive control *Six5* using SignalP even though it carries a signal peptide. A total of nine new effector genes included *ORX1* (two unique), a catalase-peroxidase (two copies), metalloprotease (two copies) and an endo-polygalacturonase (three copies) (Schmidt et al. 2013).

Other approaches for predicting both apoplastic and cytoplasmic protein effector candidates have been developed that are not entirely reliant on the previously mentioned features. Saunders et al. (2012) designed a pipeline based on fungal and oomycete effector proteins having at least one or more of the following: (i) a secretion peptide, (ii) induction of gene expression *in planta*, (iii) enriched in the haustoria, (iv) small and cysteine-rich, (v) carry a known motif or a nuclear localization signal, (vi) location on gene islands, (vii) located in regions with repeats and (viii) no PFAM domains, with exception of those known to be involved in pathogenicity. Clustering algorithms then grouped proteins into families of rust pathogens ranking them by the probability of being effectors (Saunders et al. 2012). As a result, eight families of effectors were identified. Nemri et al. (2014) applied this approach to *M. lini*, shortlisting 200 effector candidates.

Population genomic studies are valuable in identifying candidate *Avr* and effector genes, as well as evolutionary processes within pathogens (Möller and Stukenbrock 2017). Signatures of positive selection can be picked up in virulence genes (Möller and Stukenbrock 2017).

These have been studied in rust fungi (Persoons et al. 2014; Friesen et al. 2006). A genome-wide search for variation in isolates of rust fungi detected candidate *Avr* genes within their secretomes (Cantu et al. 2013; Walker et al. 2014; Persoons et al. 2014; Bueno-Sancho et al. 2017). Comparison of *Pgt* isolates with and without virulence towards *Sr50* enabled the identification of *AvrSr50* (Chen et al. 2017). AvrStb6 of *Z. tritici* was identified through a combination of QTL mapping in the progeny of two Swiss isolates and a GWAS study of around 100 isolates from a natural population from France (Zhong et al. 2017).

Various proteomics tools have been successfully utilised for large-scale studies on effector proteins. Its advantage is that it provides a direct view of cellular processes unlike genomic data analysis. Proteomics was first used in the identification of *C. fulvum* Avr9 from purified tomato apoplastic fluids using polyacrylamide gel electrophoresis, reverse-phase HPLC and EDMAN N-terminal sequencing (De Wit 1981; De Wit et al. 1986). Mass spectrometry was used in the identification of F*. oxysporum f. sp. lycopersici* Six1 (Avr3) (Rep et al. 2004). Six1 was isolated from the xylem sap of inoculated tomato plants. A gene deletion confirmed lack of the Six1 gene was associated with virulence towards plants with the *R* gene *I-3*. Other Six effectors mentioned earlier in this review were identified using proteomics of xylem sap. Limitations to the use of proteomics include low quantities of the target protein, abundance of other proteins affecting the sensitivity, resolution and speed of analysis (González-Fernández et al. 2010). It is still, however, a more specific approach for identifying effectors of interest in contrast to genomics effector prediction pipelines which shortlist effectors that might not be of interest. In this thesis, I used mutational genomics as a possible avenue to provide solutions to directly link *Avr* effectors with their corresponding NLRs (Chapter 3).

The machine learning software tool EffectorP was developed and trained on fungal effectors allowing for use to predict Pucciniaceae effectors from secretomes (Sperschneider et al. 2016b). Effector candidates are ranked by their probability to be effectors. EffectorP correctly identified positive controls *AvrL567*, *AvrP123*, *AvrP4*, *RTP1*, *AvrM14*, and *AvrL2* but not *PGTAUSPE-10-1* and *AvrM* (Anderson et al. 2016; Sperschneider et al. 2016). Cytoplasmic effectors have been found to manipulate and enter subcellular compartments and many effector candidates enter plant chloroplasts and plant nuclei (Petre et al. 2015; Petre et al. 2016). WoLF PSORT and ChloroP can be used to predict these localizations (Emanuelsson et

al. 1999; Horton et al. 2007). Challenges in effector prediction using these systems are posed by variations in signal peptide length and domains plus rapid effector evolution as predictors depend on information regarding homology (Sperschneider et al. 2017). LOCALIZER predicts effector localization to organelles such as chloroplasts, mitochondria and nuclei with higher accuracy and predicted a transit peptide for ToxA which was unknown and this was confirmed during *N. bethamiana* assays (Sperschneider, Catanzariti, et al. 2017).

1.14. Functional characterisation of effector candidates.

Multiple approaches have been used to characterise candidate effector genes. Described earlier, the host-induced gene silencing (HIGS) approach developed to silence *Pst* genes using the Barley stripe mosaic virus (BSMV) has helped evaluate genes involved in pathogenicity (Yin et al. 2011; Tang et al. 2017). The use of BSMV RNAi constructs produced siRNA molecules that targeted targeting three predicted pathogenicity genes: a MAPK, a cyclophilin, and a calcineurin regulatory subunit. Subsequent *Pt* inoculation resulted in a suppressed disease phenotype and a reduction (Panwar et al. 2013). This could be applied to verify the interaction of cloned rust *Avr* with their corresponding R genes in a cereal host background. Another interesting possibility this opens would be to determine the effect of the Avr knockdown on pathogenicity.

Overexpression of Avr and their corresponding R in a heterologous system has been widely used to verify of Avr/R interaction. The *AvrSr50-Sr50* interaction was confirmed by overexpression using a BSMV vector containing *AvrSr50* in a wheat line carrying the *R* gene *Sr50* (Chen et al. 2017). Using vectors from viruses that infect grasses enables testing of wheat rust *Avrs* on the fungal host (Bouton et al. 2018). This is useful in cases of NLRs that indirectly interact with effectors by guarding host cell target molecules which might not be present in a heterologous system such as *N. benthamiana* or Arabidopsis where transient co-expression of the *Avr-R* pair would not result in HR to confirm their interaction.

PstSCR1, and AvrSr35 (together with Sr35) induce plant cell death when expressed with their signal peptides in *N. benthamiana* (Dagvadorj et al. 2017; Salcedo et al. 2017). AvrSr35 initiated HR when injected as a purified protein into leaves of wheat carrying *Sr35* (Salcedo et al. 2017). PstSCR1 triggered non-host resistance when injected as a purified protein in *N.*

benthamiana, although its role in *Pst* is unclear (Dagvadorj et al. 2017; Salcedo et al. 2017). Diversified molecular approaches have been used in the study of *Pst* proteins involved in interaction and more than ten genes have been evaluated recently (Tang et al. 2015, 2017). In these studies, HIGS is usually employed to test shortlisted candidate genes for their role in pathogenicity. Following identification of candidate effectors, experiments to determine subcellular localization in heterologous systems or in host (wheat) protoplasts are conducted. Yeast two-hybrid assays are also used to determine effector targets (Cheng et al. 2015; Liu et al. 2014; Zhu et al. 2017; Salcedo et al. 2017; Saur et al. 2019).

For medium-throughput screens of *Pst* candidate effectors, nine candidates were screened *on N. benthamiana* and seven were shown to suppress HR (Ramachandran et al. 2017). An effector of *Pgt*, *PgtSR1* was confirmed to suppress HR in wheat through delivery by the specially designed *Pseudomonas fluorescens* EtHAn (effector to host analyser) (Upadhyaya, Ellis, and Dodds 2014). The alleles *PgtSR1-a* and *PgtSR1-b* suppress RNA silencing in plants and reduce the number of small RNAs in wheat (Yin et al. 2019). Elsewhere, localisation experiments for mature proteins of 16 *Pst* candidate effectors fused with GFP were conducted in *N. benthamiana* to determine their possible interactors by coimmunoprecipitation (coIP) and mass spectrometry (Petre, Saunders, et al. 2016). A candidate effector protein accumulated in processing bodies and interacted with an enhancer of mRNA decapping protein 4 (TaEDC4 in wheat and *N. benthamiana* (Petre, Saunders, et al. 2016).

This approach was also applied to the coffee rust fungus *H. vastatrix* to several candidate effectors that were identified by genomic and transcriptomic approaches in various studies (Fernandez et al. 2012; Cristancho et al. 2014; Talhinhas et al. 2014; Maia et al. 2017). A selection of 30 *H. vastatrix* candidate effector genes expressed in coffee leaves were assessed for their effect on the symptoms caused by *P. syringae* pv. *garcae* to determine avirulence candidates. Candidate *HvEC-016* decreased bacterial growth by suppressing HR in specific varieties (Maia et al. 2017). This, therefore, offers an opportunity to test multiple candidate effectors.

For the wheat stem rust pathogen, a high-throughput effectoromic screen was implemented using BSMV expression of a large set of candidate effectors conserved in *Pgt*, *Pst* and *Pt*. The gene *Pgt-IaaM* (tryptophan 2-monooxygenase) was discovered to play a role in auxin production and that it was required for full virulence (Yin et al. 2014). Its expression in Arabidopsis resulted in pleiotropic phenotypes that were indicative of being auxin-initiated. The use of vectors from viruses adapted to infecting cereals offers less complications in effector candidate validation than the use of a delivery system based on the non-adapted bacteria.

Efficient tools to identify and test rust effectors are still needed. Other attempts that have been tried include biolistic transient transformation of *U. fabae* with various reporters and Agrobacterium-mediated transfer of *M. lini* to silence the effector *AvrL567* (Lawrence et al. 2010; Djulic et al. 2011). Gene silencing has had more success as shown in its application in forward genetics in *Pst* to identify virulence genes (Tang et al. 2017; Cheng et al. 2017). This needs to be applied to other Pucciniales. When genomics and validation pipelines become uniform comparative studies can be done on a wider scale (Lorrain et al. 2019). These systems could also be applied to less researched areas in plant-pathogen interactions such as effector epigenetics or how pathogens extract nutrients to sustain their growth and reproduction (Lorrain et al. 2019).

1.15. Study rationale and objectives.

1.15.1. Study rationale.

In 2013, Limagrain UK identified a small, isolated *Pgt* infection in one of their breeding plots in Suffolk. Following this, a spore sample was sent to the National Institute of Agricultural Botany in Cambridge where it was bulked by our collaborator Jane Thomas. Successive rounds of single pustule increases have since been made with the isolate received from NIAB, and the resulting pure isolate has been designated as *Pgt* UK-01 (Lewis et al. 2018). Using molecular markers and whole-genome shotgun sequencing, the Saunders lab verified that *Pgt* UK-01 is indeed *Puccinia graminis* f. sp. *tritici*. Differential tests conducted by Jane Thomas in NIAB established the race of the isolate to be TKTTF according to the North American nomenclature system and with her colleagues, established that 80% of the UK's cultivars on the national Recommended List were susceptible to *Pgt* UK-01 (Lewis et al. 2018).

The simultaneous deployment of several cloned stem rust resistance genes in the wheat genome would protect wheat against *Pgt* and significantly decrease the probability of the pathogen evolving and overcoming individual Sr genes (Wulff and Moscou 2014). To achieve this a consortium of organisations serving different roles was established Involving:

• 2Blades Foundation: Overall project management including intellectual property.
• John Innes Centre: Novel *Sr* gene discovery and cloning: (*Sr22*, *Sr45*, *Sr1644*, *Sr1662*, *SrA*, *SrB iand SrC*) and *AvrSr* gene cloning.
• CSIRO: *Sr* and *AvrSr* gene cloning, stacking *Sr* genes and transforming wheat. CSIRO managed to clone *AvrSr50* which enabled the stacking of a combination of five APR and NLR genes at a single locus resulting in complete immunity against stem rust disease in wheat (2Blades Foundation 2019).
•University of Minnesota and CIMMYT: Field testing of transgenic wheat (UMN), Stacking *Sr* genes, and deployment of multi-*Sr* gene stacks in elite wheat cultivars (CIMMYT).

At the John Innes Centre, we have twelve near-isogenic lines (NILs) containing different *Sr* genes, most of which have not yet been deployed and are effective against the prevalent races of wheat stem rust; ethane methyl sulfonate (EMS)-derived mutants where the *Sr* gene has been knocked out, and the recurrent parental lines used to generate the monogenic lines (Chapter 2, Table 2.1). Out of these *Sr* genes, five have been cloned, including *Sr33* (Periyannan et al. 2013), *Sr22* and *Sr45* (Steuernagel et al. 2015), *Sr43* (Yu et al. 2020) and *Sr46* (Lagudah, unpublished), while the cloning of other Sr genes is underway within different labs in the research partnership. Gain of virulence to an *R* gene by pathogen strains can be associated with a general loss of fitness (Huang et al. 2006), thus making the use of multiple *R* genes in disease control attractive as it significantly slows down pathogen evolution towards virulence. Gene cloning and stacking opens the possibility of selection *R* genes from nonhost species that are effective against *Pgt* as has been demonstrated in disease resistance being achieved in several crop species as well as from the diverse genetic resource of wild relatives of wheat (Wulff and Moscou 2014).

Regarding this PhDs strategic interest, the main goal was:

To clone Avr(s) that are recognized by one or more cloned *Sr* genes among the twelve R genes of interest in the Wulff lab namely *Sr22*, *Sr33*, *Sr40*, *Sr43*, *Sr44*, *Sr45*, *Sr46*, *Sr51, Sr53*, *Sr1662*, *Sr1644* and *Sr2020*.

1.15.2. Specific PhD objectives

- To purify isolate *Pgt* UK-01 contaminated with *Puccinia triticina* for use in all experiments requiring the wildtype and mutant *Pgt*.

- Evaluate the monogenic lines *Sr22*, *Sr33*, *Sr40*, *Sr43*, *Sr44*, *Sr45*, *Sr46*, *Sr51, Sr53*, *Sr1662*, *Sr1644* and *Sr2020* for their resistance to wildtype *Pgt* UK-01 infection and shortlist *Sr* genes with the strongest phenotype.

- Use EMS mutagenesis to create mutant populations of *Pgt* UK-01 and determine G/C to A/T SNV transition rates at various concentrations of the mutagen.

- Screen *Pgt* mutant populations against *Sr43, Sr44* and *Sr45* and identify multiple independently derived gain-of-virulence *Avr* gene knockout mutants specific to each *Sr*.

- Establish the consistency of the virulence profile by the gain-of-virulence mutants to the wildtype *Pgt* UK-01 race TKTTF of selected mutants per set of virulent mutants for each *Sr* using differential analysis.

- Sequence the virulent mutants corresponding to *Sr43*, *Sr45* and generate a Pgt UK-01 reference assembly to clone candidate *AvrSr43* and *AvrSr45* genes.

- Conduct fitness studies of selected *AvrSr43* mutants by determining their development on *Sr43* and recurrent parent, Chinese Spring.

2. Chapter 2. Purification and initial characterisation of wild type Pgt UK-01.

The material presented in this chapter has formed the basis of the following peer-reviewed publications that I have co-authored:

1. The purification of *Pgt* UK-01 contributed to the study published in:
   Lewis, C. M., Persoons, A., Bebber, D. P., Kigathi, R. N., Maintz, J., Findlay, K., Bueno-Sancho, V., Corredor-Moreno, P., Harrington, S. A., **Kangara, N**., Berlin, A., García, R., Germán, S. E., Hanzalová, A., Hodson, D. P., Hovmøller, M. S., Huerta-Espino, J., Imtiaz, M., Mirza, J. V., Justesen, A. F., Niks, R. E., Omrani, A., Patpour, M., Pretorius, Z. A., Roohparvar, R., Sela, H., Singh, R. P., Steffenson, B. J., Visser, B., Fenwick, P. M., Thomas, J., Wulff. B. B. H., & Saunders, D. G. O. 2018. Potential for re-emergence of wheat stem rust in the United Kingdom. *Communications Biology* 1 (1): 13. https://doi.org/10.1038/s42003-018-0013-y

2. The characterisation of *Pgt* UK-01 on wheat and *Ae. tauschii* carrying single *Sr* genes including figure 2.5, Table 2.1, Annexe 1 was published in:
   Kangara, N., Kurowski, T. J., Radhakrishnan, G. V., Ghosh, S., Cook, N. M., Yu, G., Arora, A., Steffenson, B. J., Figueroa, M., Mohareb, F., Saunders, D. G. O. and Wulff, B. B. H. 2020. Mutagenesis of *Puccinia graminis* f. sp. *tritici* and selection of gain-of-virulence mutants. *Frontiers in Plant Science* 11:1-14 https://doi.org/10.3389/fpls.2020.570180

2.1 Abstract

The purification of *Pgt* UK-01 urediniospores was conducted by asexual propagation of a single uredinium/spore on a susceptible cultivar. The observation of the presence of *Puccinia triticina* (*Pt*) contamination during initial experiments necessitated the use of single pustule isolation and diagnostic PCR with markers for the genes Elongation factor-1-alpha (*TEF1_1*) and Tubulin beta chain 1 (*PGTG_12204*) (Fig 2.1). Following purification of *Pgt* UK-01, I screened 12-day-old seedlings of a panel of 13 lines for their efficacy in resisting *Pgt* infection under controlled environment conditions. These lines consisted of: (i) eleven wheat lines with chromosome segments carrying the single defined *Sr* genes (Annexe 1) from wild wheats (wheat-alien introgression lines), (ii) the wheat recurrent parents used for *Sr* introgression, (iii) two *Ae. tauschii* accessions predicted to carry only *Sr46* or *SrTA1662* (Arora et al. 2019), one *Ae*. *sharonensis* accession carrying *Sr2020*, and (iv) wheat cultivars with determined susceptibility and resistance phenotypes as experimental controls. Lines were shortlisted based on the leaf pustule size of wildtype *Pgt* UK-01 (Fig 2.2) with the largest acceptable pustule size being 2+ (moderate resistance) or smaller pustule size (2, 1+, 1 or ;) on the Stakman scale of pustule size ranging from ; (strong resistance) to 4 (susceptible) (Stakman et al. 1962). As a result, nine *Sr* genes were shortlisted for downstream experiments (Table 2.1). Mutagenized *Pgt* (Chapter 3) was screened on these *Sr* lines to identify gain-of-virulence *Pgt* mutants which were then sequenced and analysed to identify *Avr* candidates in Chapter 4.
(247 words)

2.2 Introduction.

The *Pgt* isolate used in this study was identified in the UK in 2012 (Lewis et al. 2018). This isolate, designated as *Pgt* UK-01, was identified on mature wheat plants by Limagrain UK in one of their breeding plots in Suffolk, UK (Fig 2.1 (a)) (Lewis et al. 2018). It is thought that stem rust could have been present within the UK causing sporadic infections that had no significant impact as climatic conditions that favoured its growth coincided with the mature growth stage of the wheat crop where the disease makes little impact. This was also reported in the identification of stem rust disease on Barley between mid-July and early August 2019, in a late-sown field located in Suffolk (Orton et al. 2019). The inoculum could have originated from a barberry hedge close to the field that had aecia, which were genotyped and

determined to be *Pgt* among other formae speciales (Orton et al. 2019). This highlights the decline in barberry eradication that had been implemented for centuries beforehand in the UK despite the lack of legislation like in the US (Pollard et al. 1974). The success of this was praised by Stakman, an American cereal pathologist who reported that he could not find any bushes when he surveyed Europe, whilst presenting to the USDA evidence to support the legislation of a similar programme (Stakman 1923). At present, the early maturing varieties of UK wheat and barley avoid infection. Climate change could result in a return of stem rust outbreaks which were once common. This supports the need for enhanced vigilance and monitoring across cereals and grasses in the UK. *Pgt* requires a temperature range of 17-32 ^{o}C for optimal growth and a minimum of 2 or 5 ^{o}C for spore germination or sporulation, together with long periods of dew formation on the host leaves (Singh et al. 2002).

The wheat leaf (or brown) rust pathogen, *Pt* is the most ubiquitous of the three wheat rusts (Huerta-Espino et al. 2011). *Pt* infects the leaf blades and at times the leaf sheaths under favourable conditions, heavy inoculum load or on highly susceptible cultivars. Rapid disease development is favoured by 10^{o}C and 30^{o}C which makes it present in most wheat growing areas (Browder 1971; Singh et al. 2002). Grain losses at local levels are low compared to *Pgt* and *Pst* and is mostly below 10%, however, this can reach levels above 30% when disease is severe. Globally, the overall yield losses caused by *Pt* are greater than those of *Pgt* or *Pst* because of its wider geographical distribution.

Leaf rust tends to be identified by its uredinial stage which is identified on crops in the field (Browder 1971). Uredinia are erumpent, can be as long as 1.5 mm in diameter and round to ovoid in shape (Browder 1971). Much like *Pgt*, urediniospores are the major dispersal mechanism for the pathogen. An alternative uredinia shape phenotype can be concentric rings of urediniospores (Singh et al. 2002). The urediniospores of *Pt* are orange-red or brown and the uredinia carrying them distributed on both surfaces of the leaf (Huerta-Espino et al. 2011; AHDB 2020). Because of the appearance of leaf rust disease symptoms, they can be confused with stem rust. Within a field, plants can be infected by both pathogens among others.

The lack of prevalence of *Pgt* within the UK presented an opportunity to work without risk of contamination from other *Pgt* races (Lewis et al. 2018). Isolates that would be identified on *Sr* gene monogenic lines effective in the control of *Pgt* UK-01 could be considered as gain-of-virulence EMS mutants and not contaminant isolates of other *Pgt* races with virulence on specific *Sr* lines. Wheat leaf rust, however, is widespread in the Eastern and Southern regions of the UK because of the hot, humid weather mainly in April to July, unlike in other parts of the country (AHDB 2020). Due to the ubiquity of *Pt* in the Eastern region of the UK, in which the John Innes Centre is located, I hypothesized that this could be due to contamination with *Pt*. I initially used the KWS Kielder cultivar as the susceptible line to increase my *Pgt* UK-01 spores. As a result, I had to purify my inoculum before my experiments (Fig. 2.1). To do this, I utilised single pustule isolation and PCR diagnostic markers for conserved eukaryotic genes that amplified regions that had SNPs exclusive to *Pgt* or *Pt* to differentiate several pustules that I had selected (Fig 2.1). These procedures thus enabled me to conduct routine purification of *Pgt* Uk-01 and its mutant derivatives whenever I suspected *Pt* contamination.

"Selection of *Pgt* ethyl methane sulphonate (EMS) mutants with mutations in a defined *Avr* gene (Chapter 3) requires a wheat line where the corresponding stem rust resistance (*Sr*) gene has been genetically isolated in a background which is susceptible to the *Pgt* isolate chosen for mutagenesis. The inoculation of the *Sr* line with the wildtype *Pgt* isolate determines the presence of a *Pgt Avr* corresponding to the *Sr* gene and the strength of the resistance response due to the *Sr/Avr* interaction as determined by pustule size on the leaves. The inclusion of the recurrent parent line of each *Sr* cultivar in these assays verifies if there is any additional resistance to the isolate. The presence of more than one *Sr* gene significantly reduces probability of identification of any gain-of-virulence isolates during the screens of the *Pgt* EMS mutants since mutations in multiple *Avr* will be required for an observable phenotype change from avirulence to virulence".

2.3 Results.

2.3.1 Identification of a contaminant in *Pgt* UK-01 spores used in initial experiments.

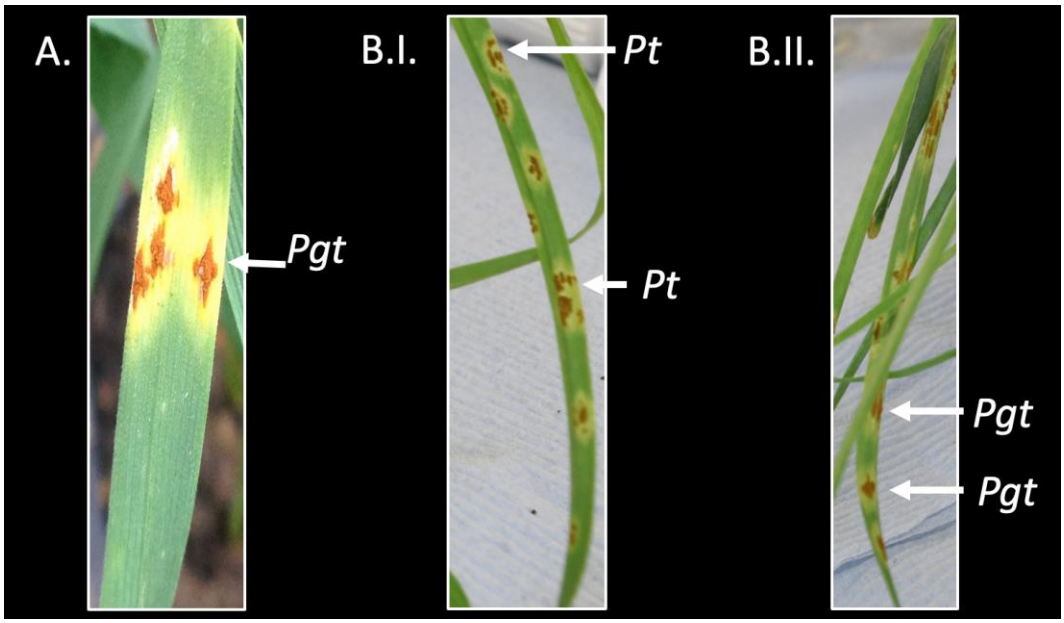


Figure 2.1 | Identification of *Pgt* UK-01 in the field at Limagrain UK and mixed pathogen uredia identified during pathogen assays at the JIC. A., *Pgt* UK-01 discovered in a breeding plot of Limagrain, UK. The wheat leaves had uredia carrying reddish-brown urediniospores (pustules) surrounded by elongated diamond-shaped chlorotic zones. Urediniospores of this field isolate were first bulked up at Limagrain and sent to NIAB. At the JIC, the cultivar KWS Kielder (+*Sr31*) was inoculated with a *Pgt* UK-01 spore ampoule received from NIAB **(B.I., B.II.)** and pustules with a leaf rust (*Pt*) morphology that were brick-red, elongated, oval and with concentric rings of spores **(B.I.)** were identified among *Pgt* pustules **(B.II.).**

Pgt UK-01 was identified on a single plant within a Limagrain breeding plot located in Woolpit village, Suffolk county, UK (Fig 2.1 A) (Lewis et al. 2018). The pustule morphology and colour was similar to commonly described stem rust disease symptoms (Singh et al. 2002). Stem rust on wheat is identified by the presence of uredinia on the plant leaves, stems, glumes, and awns. The uredinia are reddish-brown, elongated oval or diamond shaped, blister-like pustules with loosely attached spores (Agrios 2005). In the field, the leaves of wheat had uredinia (pustules) with reddish-brown asexual urediniospores that ruptured the epidermis mostly on the abaxial surface. The pustules were surrounded by a diamond-shaped chlorotic

zone (Fig 2.1 A). Paul Fenwick, a Plant Pathologist at Limagrain, identified the infection as wheat stem rust, conducted single pustule isolation of this field isolate and sent it to plant pathologists at NIAB. At NIAB in Cambridge, single pustule isolation was conducted to obtain a genetically uniform isolate for further studies. From this single pustule isolate, an ampoule of urediniospores was sent to the John Innes Centre (JIC).

At the JIC, initial bulking up was conducted using the ampule of ~50 mg of UK-01 uredinospores that was received from the National Institute of Agricultural Botany (NIAB). Inoculations were conducted on 12-day-old seedlings of the commercial winter cultivar KWS Kielder. During this process it was observed that the phenotype of the spore sample was mixed suggesting the presence of a second pathogen (Fig 2.1 B.I and B.II.). Pustules that appeared to be *Pt* were observed that were red, long oval shaped, and broke through the upper and lower leaf epidermis. The pustule patterns appeared on the abaxial and adaxial surfaces of the leaves. Some of these pustules also had concentric oval rings of urediniospores (Fig 2.1 B.I.). The pustules with morphologies like those published for *Pgt* were also present on the leaves mostly on the abaxial surface penetrating at times with a smaller area to the adaxial side (Fig. 2.1 A.II). These results suggested the presence of a second pathogen in the *Pgt* inoculations on the KWS Kielder and thus steps were taken to isolate and purify *Pgt* which was the pathogen of interest in this study.

2.3.2 Purification of contaminated *Pgt* UK-01 for use in downstream experiments.

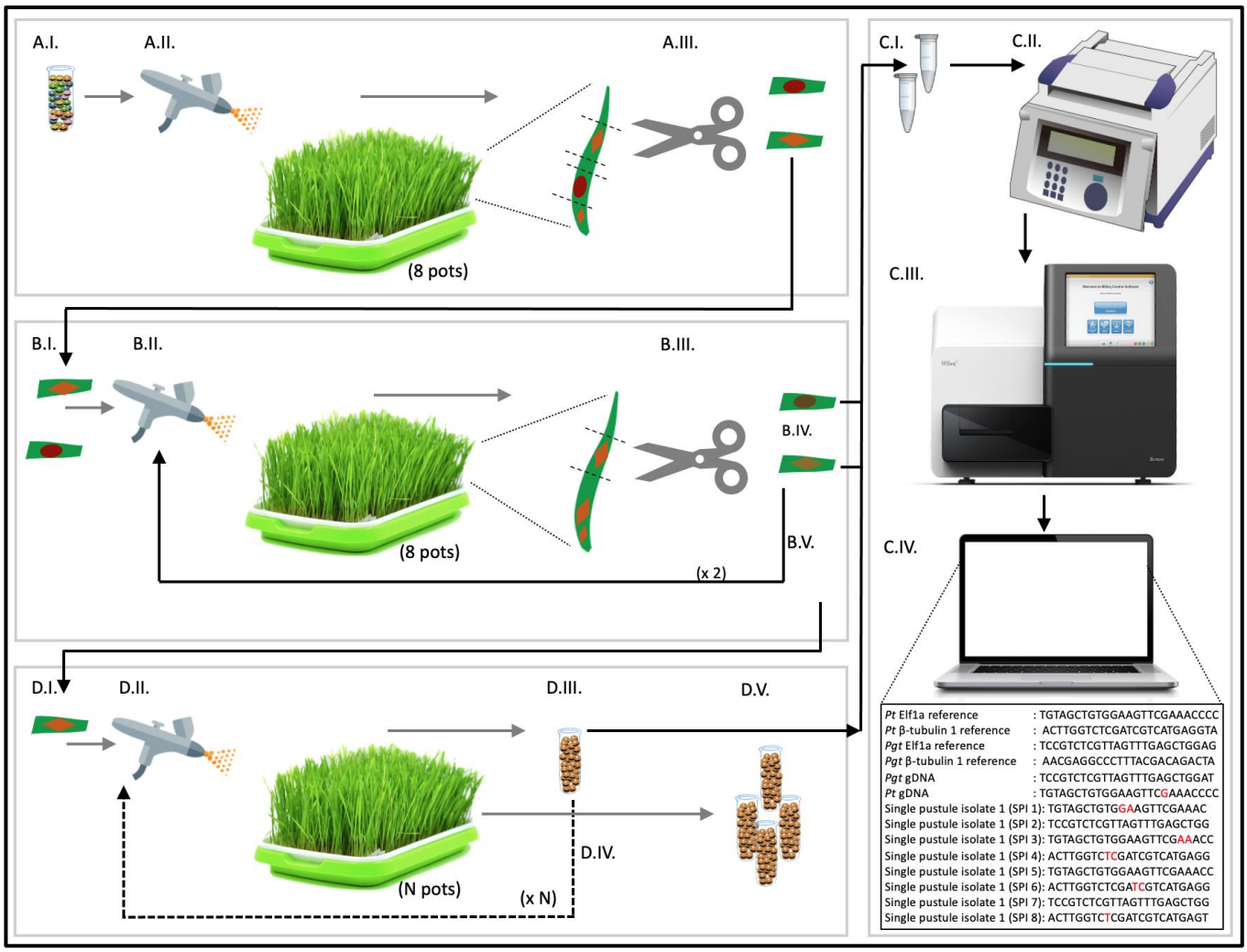
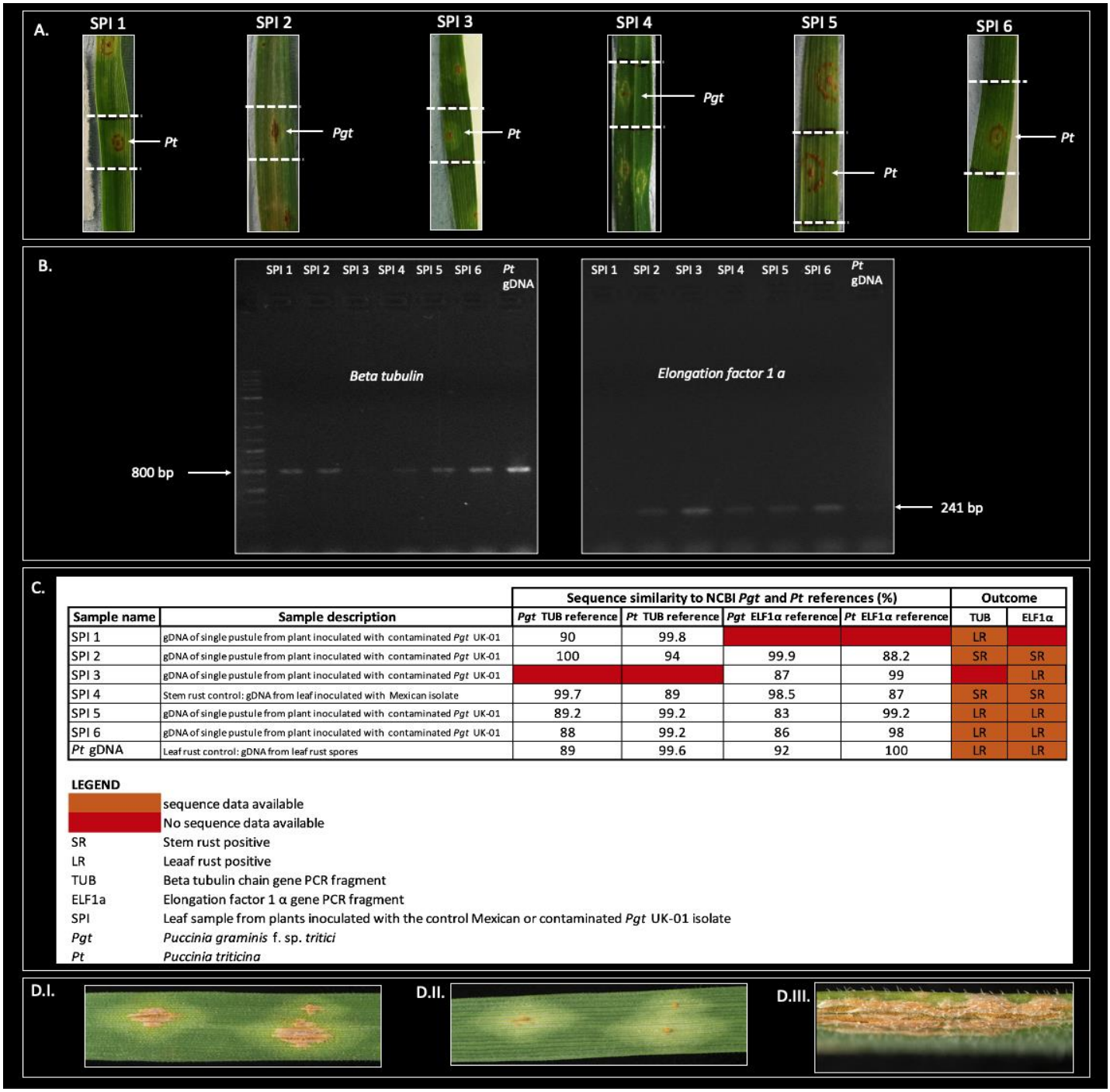


Figure 2.2 Overview of the process used to purify *Pgt* UK-01 spores contaminated with *Puccinia triticina*. The contaminated sample (**A.I**.) was suspended in NOVEC 7000^{TM} (NOVEC) and inoculated onto 8 pots containing 12-day-old seedlings of susceptible host Vuka (**A.II**.). At 12 days post inoculation, single, six well-isolated pustules with different phenotypes were harvested by cutting the leaf segment surrounding infection site and stored separately (A.III.). Each leaf segment carrying a single pustule (**B.I.**) was dipped in NOVEC 7000TM which was then applied on 8 pots of Vuka seedlings (**B.II.**). After the inoculation, the leaf segments (**B.IV.**) were used, together with controls for known Pgt and Pt, for DNA extraction (**C.I.**) followed by PCR using markers for the elongation factor-1-alpha and Beta-tubulin 1 genes (**C.II.**), sequencing (**C.III.**), and sequence analysis (**C.IV.**). Single pustules were collected from plants that were inoculated with pustules confirmed to be *Pgt* (**D.I.**). These were inoculated onto Vuka (**D.II.**) and between 21 -28 days post inoculation spores were harvested (**D.III.**) and diagnostic PCR and sequence analysis was conducted to confirm maintenance of the Pgt genotype. Upon confirmation, successive rounds of bulking up (**D.I.V.**) to obtain sufficient spores for experiments and long-term storage was conducted.

An elaborate method to differentiate between the *Pgt* and *Pt* pustules was devised (Fig 2.2). First, a halved inoculum concentration of 5 mg of the contaminated *Pgt* UK-01 urediniospores was suspended in 10 ml of NOVEC 7000^{TM} and used to spray 56 seedlings of KWS Kielder plants (Fig 2.2 A.I. – A.II). At 13 days post inoculation (dpi), well isolated single pustules with different morphologies were collected separately (Fig 2.2 A.III.). These pustules were dipped in 5 ml of NOVEC 7000^{TM} and the suspended spores were sprayed onto new seedlings (Fig 2.2 B.I. – B.II.). At 12 dpi, single pustules were collected and used to repeat the process of inoculation of fresh plants (Fig 2.2, B.III.). The remaining leaf segments (Fig 2.2 B.IV.) were used in DNA extractions (Fig 2.2 C.I.) followed by PCR of the housekeeping eukaryotic genes Beta-tubulin chain 1 and elongation factor 1 alpha using primers that amplified regions that were polymorphic between *Pgt* and *Pt* (Fig 2.2 C.II.). The PCR products were sequenced (Fig 2.2 C.III.) and aligned to reference gene sequences from NCBI (Fig 2.2 C.IV.). New pustules were collected from plants that were inoculated with single uredinia that was genotyped as *Pgt* to initiate the bulking up of the purified isolate (Fig 2.2 D.I – D.II). Further bulking up of the purified *Pgt* UK-01 was conducted using harvested spores (Fig 2.2 D.III – D.IV). DNA was extracted from the spores for genotyping as for the single pustules to confirm the success of the purification process.



C.

Sample name	Sample description	Sequence similarity to NCBI *Pgt* and *Pt* references (%)				Outcome	
		Pgt TUB reference	*Pt* TUB reference	*Pgt* ELF1α reference	*Pt* ELF1α reference	TUB	ELF1α
SPI 1	gDNA of single pustule from plant inoculated with contaminated *Pgt* UK-01	90	99.8			LR	
SPI 2	gDNA of single pustule from plant inoculated with contaminated *Pgt* UK-01	100	94	99.9	88.2	SR	SR
SPI 3	gDNA of single pustule from plant inoculated with contaminated *Pgt* UK-01			87	99		LR
SPI 4	Stem rust control: gDNA from leaf inoculated with Mexican isolate	99.7	89	98.5	87	SR	SR
SPI 5	gDNA of single pustule from plant inoculated with contaminated *Pgt* UK-01	89.2	99.2	83	99.2	LR	LR
SPI 6	gDNA of single pustule from plant inoculated with contaminated *Pgt* UK-01	88	99.2	86	98	LR	LR
Pt gDNA	Leaf rust control: gDNA from leaf rust spores	89	99.6	92	100	LR	LR

LEGEND

	sequence data available
	No sequence data available
SR	Stem rust positive
LR	Leaaf rust positive
TUB	Beta tubulin chain gene PCR fragment
ELF1a	Elongation factor 1 α gene PCR fragment
SPI	Leaf sample from plants inoculated with the control Mexican or contaminated *Pgt* UK-01 isolate
Pgt	*Puccinia graminis* f. sp. *tritici*
Pt	*Puccinia triticina*

Figure 2.3 | **Purification of *Pgt* UK-01 to bulk up for subsequent experiments.**

To purify the *Pgt* isolate, KWS Kielder seedlings (+*Sr31*) were inoculated with the *Pgt* urediniospores contaminated with *Pt* urediniospores. **A.,** Single, well isolated pustules labelled SPI 1 to SPI 6, with varying morphologies were collected separately. These single pustules were then used to inoculate susceptible Vuka seedlings (-*Sr31*). **B.** DNA was extracted from the remaining leaf segments and PCR amplification of polymorphic regions of the elongation factor-1-alpha and Beta-tubulin chain 1 genes was conducted for the DNA

samples (lane numbers correspond with SPI numbers) in addition to the *Pt* positive control labelled *Pt* gDNA. **C.** The sequenced PCR fragments were aligned to gene reference sequences for: *Pgt* Beta tubulin chain 1 (NCBI accession XM_003330619.2), *Pgt* elongation factor-1-alpha (NCBI accession XM_00333302.4), *Pt* Beta-tubulin chain 1 (NCBI accession EF570812.1) *Pt* elongation factor-1-alpha (NCBI accession JX533509.1). SPI 2 was genotyped as *Pgt* based on its higher sequence identity to the *Pgt* reference genes for Beta-tubulin chain 1 and elongation factor 1 alpha (100 % and 99 %) compared to its identities to the *Pt* references (84 % and 88.2 %). The Mexican *Pgt* isolate positive control SPI 4 had a higher sequence similarity to the *Pgt* Beta-tubulin chain 1 and elongation factor-1-alpha references (99.7 % and 98.5 % respectively) compared to the those for the *Pt* references (89 % and 87 %). The *Pt* positive control showed 99.6 % and 100 % identities the *Pt* references compared to the *Pgt* references (89 % and 92%). SPI 1, 3, 5 and 6 were identified as *Pt*. **D.**, The phenotype of the purified *Pgt* UK-01 (SPI 2) inoculated onto the susceptible wheat cultivar Vuka (-*Sr31*). **D.I.,** Individual reddish-brown pustules of *Pgt* UK-01 surrounded by diamond-shaped chlorotic areas on the abaxial leaf surface, **D.II.**, Smaller area of pustule emergence on the adaxial leaf surface, however, the chlorotic area was still visible, **D.III.**, Close-up picture of erumpent pustules emerging from the mesophyll layer through the leaf epidermis.

To obtain a pure culture of *Pgt* UK-01 urediniospores from the contaminated spores, leaf segments containing single pustules (SPI) were collected from KWS Kielder plants (+*Sr31*) inoculated with the *Pgt* UK-01 spore sample received from NIAB (Fig 2.3 A). The pustules that were collected had varying morphologies. Some pustules were found to be elliptic or had concentric circles which is not a phenotype typical of *Pgt*, as shown by the examples of SPI 1, 3, 5 and 6 (Fig 2.3 A). SPI 1, 3, and 6 were collected from plants inoculated with the UK-01 urediniospores received from NIAB. SPI 5 was collected from plants inoculated with the *Pgt* positive control which is a *Pgt* isolate from Mexico.

The pustules that had morphologies more consistent with what is known for *Pgt* were SPI 2, from inoculations made using the UK isolate and SPI 4 obtained using the *Pgt* positive control isolate (Fig 2.3 A). These had the diamond-shaped pustule morphology. In both inoculations, atypical uredinia morphologies suspected to be consistent with *Pt* infection could also be seen in inoculations made with the isolate. SPI 2 and 4 were restricted in their size and there were diamond-shaped necrotic regions surrounding the pustule as shown by SPI 4 (Fig 2.3 A). The cultivar Kielder is suspected to carry *Sr31* which is effective against UK-01 and other isolates (Lewis et al. 2018). Paul Fenwick, a Plant Pathologist at Limagrain, realized this and therefore advised us to use another cultivar (Personal communication).

To confirm the identity of the causal pathogens for the phenotypes observed during inoculations with the UK-01 and Mexican isolates, DNA was isolated from each leaf segment after retrieving the urediniospores they carried. Following this, PCR primer pairs were designed by Jens Maintz corresponding to the genes for elongation factor-1-alpha and Beta-tubulin chain 1. SPI 4, the *Pgt* positive control from inoculations made with a Mexican isolate tested positive for stem rust with high sequence identity to *Pgt* Beta-tubulin chain 1 (Fig 2.3 B). The identity of each sample was confirmed by sequencing the PCR products followed by BLAST search on NCBI to determine their identity via percentage similarity. SPI 2 was genotyped as *Pgt* based on its higher sequence identity to the *Pgt* reference genes for Beta-tubulin chain 1 and elongation factor 1 alpha (100 % and 99 %) compared to its identities to the *Pt* references (84% and 88.2%) (Fig 2.3 C). The Mexican *Pgt* isolate positive control SPI 4 had a higher sequence similarity to the *Pgt* Beta-tubulin chain 1 and elongation factor-1-alpha references (99.7% and 98.5% respectively) compared to the those for the *Pt* references (89% and 87%) (Fig 2.3 C). The *Pt* positive control showed 99.6% and 100% identities to the *Pt* references compared to the *Pgt* references (89% and 92%) (Fig 2.3 C). SPI 1, 3, 5 and 6 were identified as *Pt* based on their higher % similarity of their resultant PCR products to *Pt* as opposed to *Pgt* (Fig 2.3 C).

Inoculations on Vuka with purified *Pgt* UK-01 spores extracted from SPI 2 showed a significant increase in pustule size of *Pgt* (Fig 2.3 Di – Diii.) compared with its growth on KWS Kielder which exhibited resistance to SPI 2 (*Pgt* UK-01) and SPI 4 (Mexican isolate) in Fig 2.3 A, likely due to the presence of *Sr31* (Paul Fenwick, personal communication). A diamond-shaped chlorotic region was observed unlike a zone of necrosis due to the hypersensitive resistance response.

2.3.3 Selection of *Aegilops tauschii* lines carrying only *Sr45*, *Sr46* or *SrTA1662.*

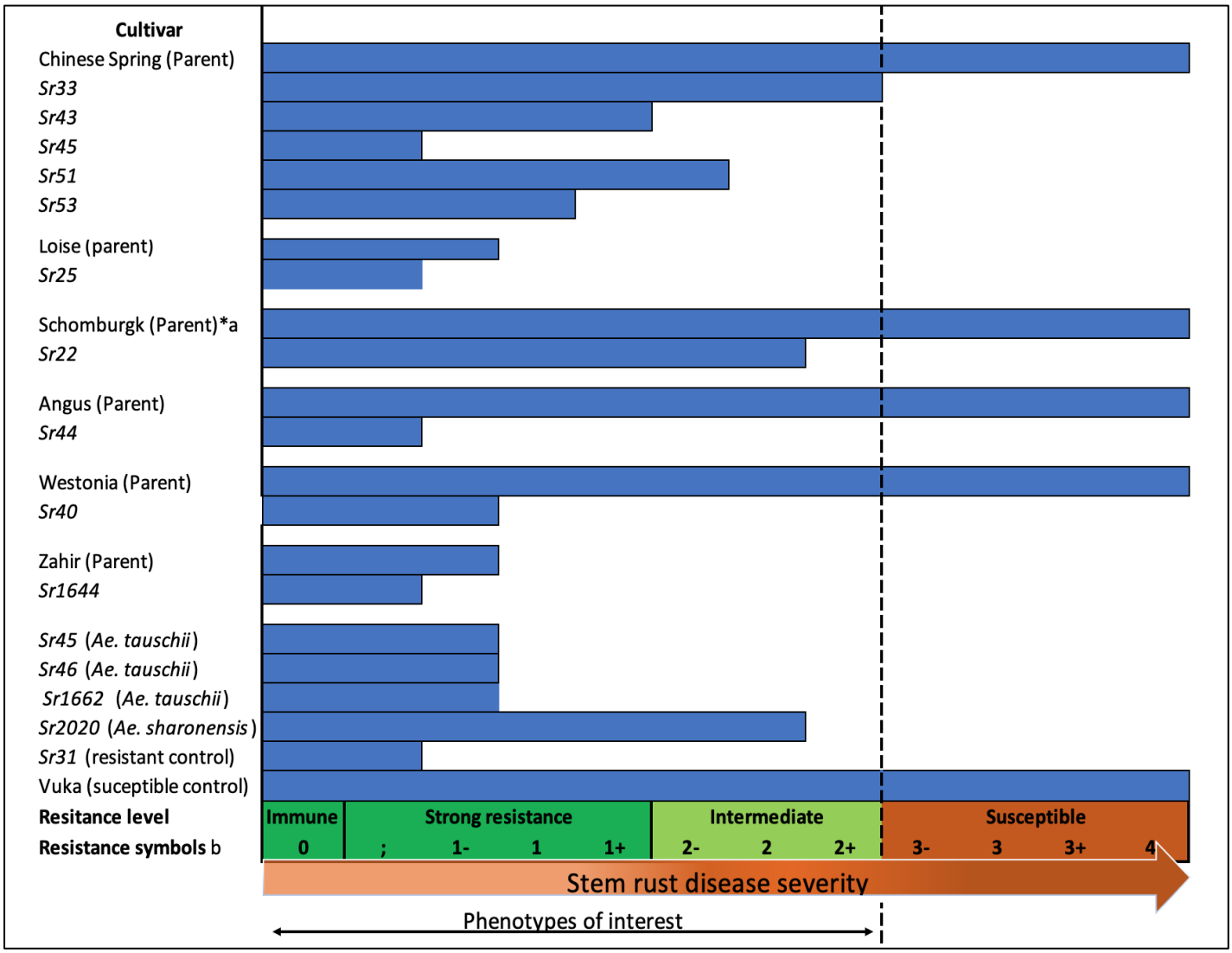
Ae. tauschii lines carrying the cloned *Sr* genes *Sr45*, *Sr46* and *SrTA1662* were shortlisted for possible screening with the mutant populations generated in Chapter 3 (Steuernagel et al. 2016; Arora et al. 2019). To select the lines carrying only *Sr45*, *Sr46* or *SrTA1662*, Dr Sanu Arora at the JIC carried out a BLAST search (using a ≥99.8% identity and >90% query coverage

cut-off) to determine the occurrence of these genes in an array of 151 *Ae. tauschii* accessions whose NLR repertoires had been captured and sequenced via nucleotide binding and leucine-rich repeat domain (NB-LRR) gene-specific enrichment and sequencing (Arora et al. 2019). As a result, she identified accessions carrying only one of the three genes namely, TOWWC191 for *Sr45*, TOWWC152 for *Sr46* and TOWWC017 for *SrTA166*. These accessions had been previously screened with *Pgt* UK-01 and found all the lines to be resistant (Arora et al. 2019). These accessions are available from the Germplasm Resource Unit at the John Innes Centre, UK.

2.3.4 Characterisation of *Pgt* UK-01 on wheat and *Ae. tauschii* carrying single *Sr* genes.

The lines used in these experiments were sourced from researchers that transferred *R* genes derived from wheat wild relatives into wheat lines. The nature of these lines and their source is explained in Annexe 1. These resistance genes except for *Sr22* and *Sr25*, are not yet deployed in cultivated wheat. During their development, they were tested and found to be effective against prevailing *Pgt* races. To determine whether *Pgt* resistance by each introgression line (Table 2.1) was conferred by its alien chromatin segment, we inoculated the purified *Pgt* UK-01 onto nine wheat *Sr* introgression lines, their corresponding six wheat recurrent parents, two *Ae. tauschii* lines (Arora et al. 2019) and one *Ae. sharonensis* line carrying single *Sr* genes and two susceptible wheat checks. The resistance of the lines against *Pgt* UK-01 provided a basis for selection of the most effective *Sr* genes with an infection type (IT) of 2+ or less against the isolate and provided a reference of the baseline resistance score to enable identification of virulent *Pgt* mutants (Fig 2.4). The phenotypes were assessed system developed by Stakman et al. (1962). Infection types or classes were denoted 0 (immune, no symptoms). ; denotes (hypersensitive necrotic lesions no pustules), 1 (very resistant, small pustules present), 2 (moderate resistance with small to medium sized uredia), 3 (moderate susceptibility exhibiting medium sized uredia), 4 (complete susceptibility with large uredia) and X (heterogenous with all phenotypes present). Intermediate pustule sizes were shown with the negative symbol – (smaller than typical uredia for the size category) or positive + (smaller than typical pustules within the size category). Where two phenotypes were present on the same plant, their sizes would be noted starting with the most abundant. A full description of this system, with a pictorial guide, is outlined in Materials and Methods

section 2.5.2. According to this scale, avirulent/ resistant designations range from 0 to 2+ whilst the symbols 3 and 4 are considered virulent.



*Seed not available

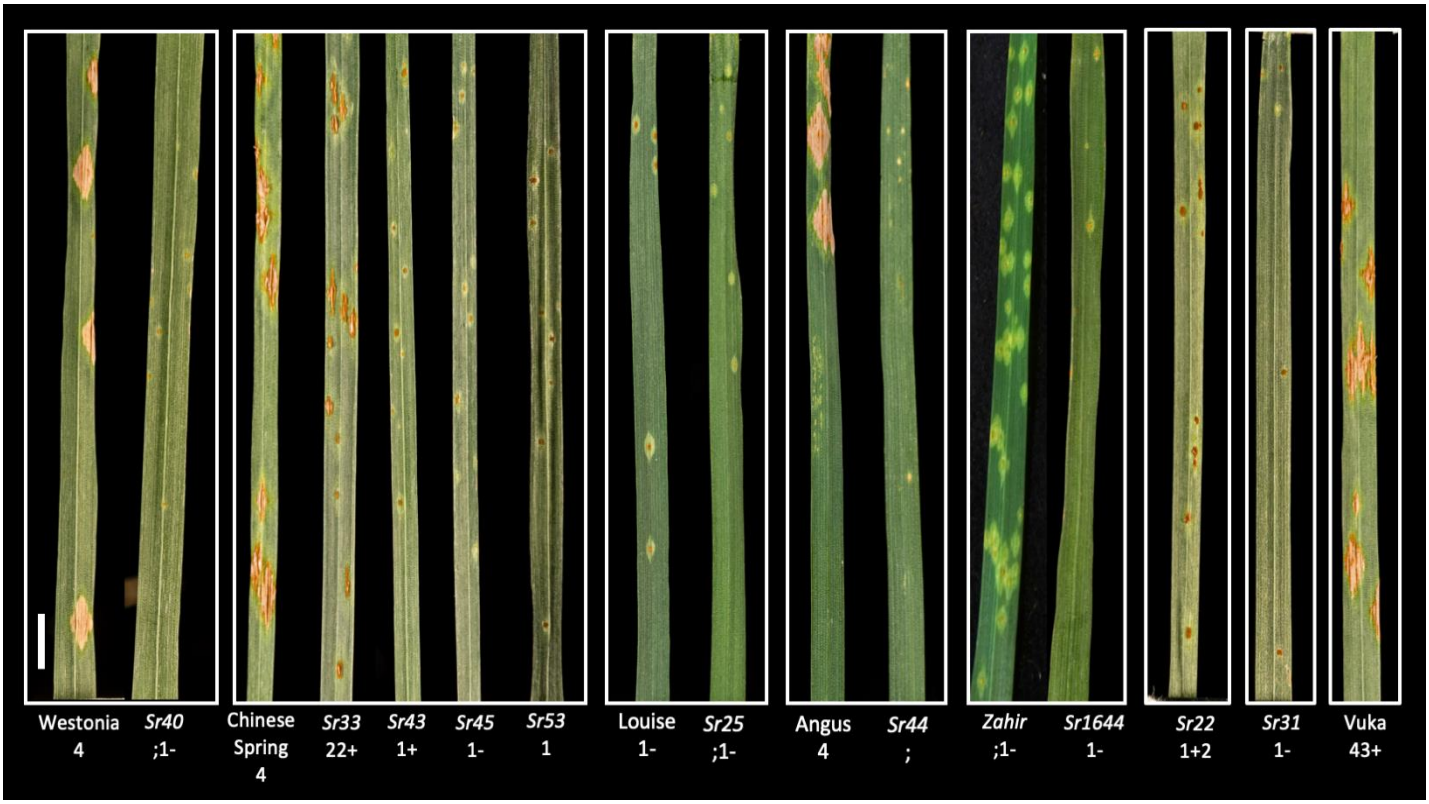
a. Phenotype according to Steuernagel *et al.* (2016).

b. Internationally accepted stem rust disease phenotype designations (Stakman and Levine 1922).

Figure 2.4 | The criterion for shortlisting *Sr* genes effective in controlling *Pgt* UK-01 infection. Introgression lines and their recurrent parent lines were inoculated with UK-01 to determine their suitability for use in downstream experiments to identify gain-of-virulence *Pgt* mutants. The lines were selected based on their strong resistance in the introgression line and susceptibility of the recurrent parent. This was based on the phenotyping scale which denotes resistance phenotypes ranging from the strongest resistance reaction of immune presented as 0 (no visible symptoms) to susceptible, which has the largest pustule size and is termed as 4 (Stakman et al. 1962). Monogenic *Sr* lines with a pustule size of 2+ or less (bars indicating pustule type that ending at or before the dotted cut-off line) with their recurrent parent lines being susceptible (sizes 3 to 4) were selected for further experiments. The stem

rust severity is indicated by Stakman (1962) symbols for pustule size and is not quantitative. Bars show pustule classification. At least two biological replicates were conducted with five seedlings per cultivar in each experiment

All ten wheat-alien introgression lines and three *Aegilops* accessions exhibited an acceptable resistance response of 2+ or less (Fig 2.4). The *R* gene *Sr44* showed the strongest phenotype and fully suppressed pustule development resulting in a fleck infection type (IT ;). Thus, nine *Sr* lines, namely *Sr22* (IT 1+2), *Sr33* (IT 22+), *Sr40* (IT ;1-), *Sr43* (IT 1+), *Sr44* (IT ;), *Sr45* (IT 1-), *Sr53* (IT 1), *Sr2020* (IT 21) and *SrTA1662* (IT 1-), were shortlisted for experiments in cloning of avirulence effectors (Figures 2.4 and 2.5; Table 2.1). *Sr51* was not included in the shortlist due to physiological issues regarding spontaneous leaf chlorosis at 12 dpi. In addition, *Sr1644* and *Sr25* were not considered candidates for *Avr* cloning as their recurrent parents Louise and Zahir both exhibited IT 1+. This indicated that the resistance exhibited by their progenies was not solely due to the presence of *Sr25* or *Sr1644* respectively. As a result, during *Pgt* mutant screens, it would be statistically improbable to get multiple independently derived gain-of-virulence *Pgt* mutants which would have lost more than one effector.



Reproduced from Kangara et al. (2020)

Figure 2.5 | **Infection types (Stakman et al. 1962) of *Pgt* UK-01, race TKTTF against nine *Sr* genes.**

Pgt UK-01 infection types scored at 14 days post inoculation (dpi) on a panel of wheat recurrent parents and their wheat-alien introgression progeny carrying single defined *Sr* genes, plus resistant *Sr31* (cv. Kavkaz/Federation4) and susceptible (cv. Vuka) control lines. Lines were scored for infection types (ITs) using the 0-4 scale (Stakman et al. 1962). *Sr40* exhibited strong resistance as opposed to its recurrent parent Westonia, which was susceptible. *Sr33* exhibited moderate resistance, while *Sr43*, *Sr45* and *Sr53* displayed strong resistance as opposed to their recurrent parent Chinese Spring, which was susceptible. Although *Sr25* showed strong resistance, its recurrent parent Louise also exhibited strong resistance. *Sr44* and its recurrent parent Angus displayed distinct resistant and susceptible ITs. Both *Sr1644*-1sh and its recurrent parent Zahir were resistant. *Sr22* was resistant whilst its recurrent parent Schomburgk (not shown) was susceptible. *Pgt* UK-01 was avirulent on the resistant control *Sr31*. Introgression lines are grouped in the same box with their recurrent parents except for *Sr22* and *Sr31*. The size bar represents 1 cm. At least two biological replicates were conducted and five seedlings per cultivar in each experiment.

Table 2.1 | Summary of *Pgt* UK-01 infection types observed at 14 days post inoculation (dpi).

***Sr* gene**	***Sr* gene source**	**Infection type**	**Recurrent parent**	**Recurrent parent infection type**	**Selected for further analysis**
***Sr22**	*Triticum monococcum*	1+2	Schomburgk	Not tested	Yes
Sr25	*Thinopyrum ponticum*	;1-	Louise	1-	No
Sr33	*Ae. tauschii*	22+	Chinese Spring	4	Yes
Sr40	*T. timopheevii* ssp. *armeniacum*	;1-	Westonia	4	Yes
Sr43	*Th. ponticum*	1+	Chinese Spring	4	Yes
Sr44	*Th. intermedium*	;	Angus	4	Yes
Sr45	*Ae. tauschii*	1-	Chinese Spring	4	Yes
Sr46	*Ae. tauschii*	1-	No parent	No data	No
Sr51	*Ae. searsii*	1+2-	Chinese Spring	4	Yes
Sr53	*Ae. geniculata*	1	Chinese Spring	4	Yes
Sr1644	*Ae. sharonensis*	1-	Zahir	;1-	No
Sr1662	*Ae. tauschii*	1-	No parent	No data	No
Sr2020	*Ae. sharonensis*	21	No parent	No data	No
***Sr31* (resistant control)**	*S. cereale*	1-	Kavkaz	Not tested	No
Vuka (susceptible control)		32+	No parent	No data	No

**Sr22* was selected for further testing based on the susceptibility of Schomburgk to *Pgt* determined by Steuernagel et al. (2016).

The phenotype of UK-01 on the thirteen target single *Sr* genes carried either by wheat or Aegilops lines that were investigated met the cut off criteria of IT ranging from ; (fleck) which is strong resistance, to 2+ (intermediate resistance) (Stakman et al. 1962). The only introgression line that did not meet the selection criterion was *Sr53* (2+3-) and hence was not a candidate for cloning its corresponding *Avr* from UK-01. Among the shortlisted genes *Sr22*, *Sr45* (Steuernagel et al. 2016), *Sr33* (Periyannan et al. 2013), *Sr43* (Yu, unpublished), *Sr46*

(Arora et al. 2019; Gaurav et al. 2021) and *SrTA1662* (Arora et al., 2019) have been cloned. The availability of cloned *Sr* genes presents an opportunity for faster functional validation of a candidate *Avr* genes by transient co-expression of the *Sr* gene and *Avr* gene pair in a heterologous system like *N. benthamiana.* However, *SrTA1662* was removed from the shortlist because of its limited range of effectiveness against other prevalent *Pgt* isolates (Arora et al. 2019). Also, whilst resistance conferred by *Sr46* was strong in the *Ae. tauschii* background, it was shown to be weak when expressed in wheat and under low or high temperature conditions (Evans Lagudah, unpublished) so this gene was also removed from consideration.

2.4 Discussion.

The presence of *Pt* contamination in the *Pgt* urediniospore sample received from NIAB meant that prior to determining the *Sr* lines effective in controlling *Pgt* UK-01, the *Pgt* UK-01 sample required purification. Reddish brown oval shaped pustules with concentric circles which are indicative of *Pt* could be identified among dark red-brown diamond shaped pustules which are in line with the stem rust phenotype. The presence of *Pt* would have made phenotyping of *Pgt* on the chosen *Sr* lines unreliable as the phenotype of the two pathogens was difficult to differentiate at times. The molecular markers designed to differentiate between *Pgt* and *Pt* proved reliable. The two sets of primers designed were specific to the conserved eukaryotic genes Elongation factor-1-alpha and Tubulin Beta chain 1 and amplified regions of the genes where natural variation occurs that is specific to each pathogen. BLAST analyses of fragments amplified from DNA extracted from pustules used in single pustule isolation determined whether they were *Pgt* or *Pt* with at least 100% and 99.7% identity respectively in the case of Tubulin Beta chain 1. The percentage identities were significantly lower when *Pgt*-positive fragments were aligned to *Pt* reference sequences and vice-versa.

The prevalence of leaf rust in the UK might have made it possible for the UK-01 spore sample to be contaminated in the field where it was discovered at Limagrain since field plants usually get infected by more than one pathogen. Alternatively, the plants used in the single pustule isolation process to purify the isolate could have been contaminated with leaf rust from the environment. This might be due to this work being carried out over summer, when leaf rust is prevalent due to the favourable warm conditions, through to the onset of winter.

The initial attempts to purify the sample obtained from NIAB using cv. KWS Kielder might have selected for leaf rust as it was later realised that this cultivar contains *Sr31* and is therefore resistant to *Pgt* UK-01. Characterisation of European *Pgt* isolates from 2013 and 2014 by differential tests and phylogenetic analyses revealed that most of them are related to the "Digalu" race (Olivera et al. 2015a) which could have originated in Turkey and spread to Ethiopia via the Middle East and back into Europe (Meyer et al. 2017b). The *Pgt* UK-01 race was determined to be TKTTF using the method of characterisation of the physiological races of *Pgt* (Stakman et al. 1962; Jin et al. 2008; Lewis et al. 2018). This race type was determined

by the isolate's virulence profile when inoculated on the stem rust international differential set which contains 20 lines carrying single defines *Sr* genes (Stakman et al. 1962). The isolate is closely related to other European isolates that were identified in Denmark (2013), Sweden (2014) and in Ethiopia in 2014 and 2015 (Lewis et al. 2018). I confirmed that the wildtype isolate I bulked up and used in my experiments maintained the TKTTF virulence profile in the differential tests I conducted in Chapter 4. Unlike Ug99, the Digalu lineage is avirulent on *Sr31* (Olivera et al. 2015a; Patpour et al. 2016).

Introgression lines carrying single *Sr* genes that had not yet been cloned such as *Sr1644*, *Sr25* and *Sr51* were resistant to UK-01 were not shortlisted. This was because both Zahir and Louise, the recurrent parents of introgression lines carrying *Sr1644* and *Sr25* respectively had an IT of 1+2- which was indicative of the presence of background resistance. Additional resistance in the recurrent parents would significantly reduce the probability of obtaining gain-of-virulence *Pgt* ethyl methanesulfonate mutants (Chapter 3) for cloning *Avr* genes corresponding to *Sr* genes. *Sr25* is the only gene in the panel that has been deployed in cultivars and natural *Pgt* isolates exist that have overcome its resistance. The *Sr51* introgression line was also discounted from further investigations as it was physiologically unstable. General leaf chlorosis in this line occurred close to the time of phenotyping the rust infection such that assessment of infection could be done on very few leaves on the seedlings.

The successful purification of UK-01 in this chapter enabled its bulking up for use in future experiments, leading towards the cloning of *Avrs* (Chapters 3 and 4). The profiling of its virulence on the *Sr* lines of interest along with their recurrent parents here produced a shortlist of eight *Sr* lines for consideration. Following these findings, it was possible to commence EMS mutagenesis of UK-01 and screening on *Sr* lines for multiple independently derived gain-of-virulence mutants.

2.5 Materials and Methods.

2.5.1 Single pustule isolation and purification of *Pgt*_UK-01.

I initially prepared sixteen pots of 12 to 14-day-old wheat seedlings of cv. KWS Kielder by sowing seven wheat seedlings per 9 x 9 cm pot. The seedlings were grown in a controlled environment room with conditions with 16 h light, 23 ^{o}C and 8 h dark, 15 ^{o}C cycle. Each pot was treated with 50 ml of 0.2 g/l maleic hydrazide upon emergence of the leaf coleoptile on the seedlings which occurs at approximately day six. The seedlings were then inoculated at 12 days post emergence using the *Pgt* urediniospores contaminated with *Pt* urediniospores. The dried urediniospores used in the inoculations were supplied by NIAB and were stored at -80 ^{o}C. The spores were first heat-shocked by placing them in vials in a water bath set at 45 ^{o}C for 15 minutes. Following this, the spores were suspended in 3M™ Novec™ 7000 Engineered Fluid (Novec) at a rate of 4 mg of spores in 10 ml Novec per eight pots (56 plants) using an airbrush. Upon inoculation, plants were misted in sealed biological waste bags with a small amount of water at the bottom whilst under dark incubation for 24 hours. After this, each pot was covered with a 180 x 300 mm transparent cellulose film cross-bottom bag (Helmut Schmidt Verpackungsfolien GMBH) and grown under controlled conditions described above.

From 14 DPI onwards, single pustule isolation and genotyping was conducted by selecting well developed and isolated pustules that were first phenotyped, cut from the leaves and placed in separate airbrush vials with approximately 5 ml of Novec for inoculation onto 12-day-old Vuka seedlings. After inoculation, the remaining leaf segments in the airbrush vials were transferred into 2 ml centrifuge tubes with 70% ethanol and stored at -20 ^{o}C before genotyping. DNA extraction from the infected leaf segment used in single pustule isolations was carried out using a Qiagen^{TM} DNeasy^{TM} plant mini kit and DNA was quantified with a NanoDrop™ 2000 spectrophotometer. PCR was conducted using two primer sets (Table 2.2) separately for each DNA sample using the program detailed in Table 2.3.

Table 2.2 | **PCR primers used to amplify target genes.**

Gene	Forward primer	Reverse primer	Length of region
PGTG_12204 (Tubulin beta chain 1)	CCGATCTGGCGCGTTTG	GTCAAGTATCGGCCGTGTC	816 bp
TEF1_1 (Transcription elongation factor 1α)	CCAGAAGGTCGCCATCAAG	ATACCAGCTTCGAATTCACCA	245 bp

Table 2.3 | **PCR conditions used to amplify target genes**

Step	Temperature	Time
Initial denaturation	95 °C	5 minutes
35 cycles	95 °C	30 seconds
	59 °C	20 seconds
	72 °C	60 seconds
Final extension	72 °C	5 minutes
Hold	18 °C	Optional

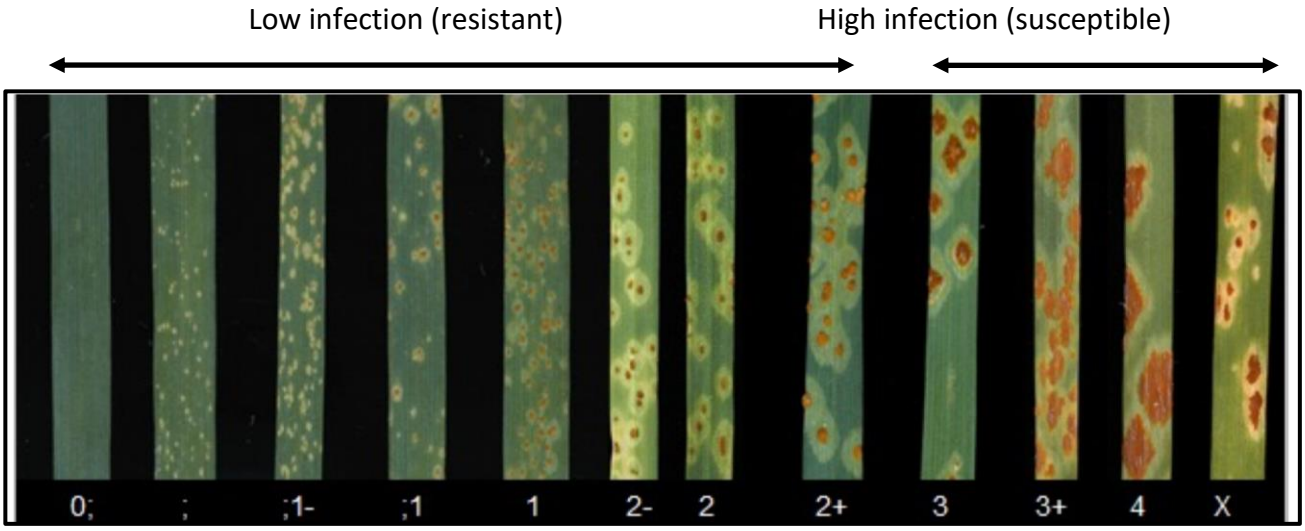
Following PCR, the product was purified using QIAquick™ PCR purification kit. DNA was quantified by gel electrophoresis of Enterobacter lambda λ DNA aliquots loaded onto a 1 % agarose gel followed by DNA quality analysis using a NanoDrop™ 2000 spectrophotometer. Samples with amplified DNA were sent for Sanger sequencing at GATC™. Reads were trimmed and BLAST searches carried out on the National Center for Biotechnology Information (NCBI) database within Geneious R10. Consensus files were viewed in Geneious™ R10.

2.5.2 Bulking up of purified *Pgt* UK-01 urediniospores and determining its infection type on target *Sr* lines.

A single pustule that was well separated from other pustules and which exhibited the stem rust phenotype was harvested and used fresh to inoculate eight pots of Vuka seedlings using the process previously described for seedling preparation and inoculation. At the onset of sporulation, the single pustule isolation process was repeated. The seedlings were grown in a controlled environment room with conditions as previously described.

The spores were collected three weeks after the onset of sporulation. Spores were dried for four days by placing in vials which were plugged with cotton wool in sealed containers with silica beads. The spores were then stored at -80 ^{o}C for future use.

Seedlings of *Sr* introgression lines, their recurrent parents, and *Ae*. *sharonensis* and *Ae. tauschii* lines (Table 2.1) were inoculated using purified UK-01. Spores were taken from storage at -80 ^{o}C and were first heat shocked before use. The inoculum used was 10 mL of Novec containing 10 mg of spores per eight pots (64 plants). Phenotyping was done at 14 DPI using the Stakman et al., (1962) method shown below in Fig 2.5.



(Woldeab et al. 2017)

Figure 2.6 | **Pustule classification that was used in all phenotyping experiments which was based on the Stackman (1962) system.** Infection types or classes were denoted 0, ;, 1, 2, 3, 4 and X. ; denotes hypersensitive flecks, necrotic lesions are observed and no pustules are present. 0 - Immune, no uredia develop; hypersensitive flecks might also be present. 1 (Very resistant) the uredia are small and spaced; bounded by hypersensitive reaction. 2 - Moderate resistance showing isolated uredia that are small to medium in size along with hypersensitive areas that look like halos or circles. The pustules are sometimes surrounded by green areas that are slightly chlorotic. 3 - Moderate susceptibility, uredia are medium sized, these sometimes coalescence. Sporulation is below normal. There are no true hypersensitive flecks but mainly chlorotic regions. 4 - Complete susceptibility with numerous, large, and merging uredia. No hypersensitive reaction. X - Heterogenous phenotype, high variation of uredia size showing all types of reactions including intermediates.

Fluctuations within a class have been designated by plus and minus signs. For some pustules, when present, symbols – and + were added after the number denoting pustule type. – represented slight rust development where the pustule formation was below what is considered normal, but still somewhat better than the size preceding it. + was added to a pustule phenotype when infection size was considered better than normal but still less than the size following it on the scale. Uredia denoted + were also fairly numerous and scattered. When more than one pustule size was observed on a plant two symbols were recorded for starting with the predominant size as well as the intermediate reaction e.g. 22+ (two and two-plus) showing a phenotype composed of mainly size 2 along with a smaller number of larger, 2+ sized pustules.

The material presented in this chapter has formed the basis of the following peer-reviewed publication that I co-authored:

Kangara, N., Kurowski, T. J., Radhakrishnan, G. V., Ghosh, S., Cook, N. M., Yu, G., Arora, A., Steffenson, B. J., Figueroa, M., Mohareb, F., Saunders, D. G. O. and Wulff, B. B. H. 2020. Mutagenesis of *Puccinia graminis* f. sp. *tritici* and selection of gain-of-virulence mutants. *Frontiers in Plant Science* 11:1-14 https://doi.org/10.3389/fpls.2020.570180

3. Chapter 3. Optimising an EMS mutagenesis procedure for *Pgt* and selection of isolates with gain-of-virulence to *Sr43* and *Sr45.*

3.1 Abstract

The development and deployment of wheat cultivars with multiple stem rust resistance (Sr) genes is a strategy that could provide durable resistance to infection by *Pgt*. However, certain disease resistance genes negatively interact or fail to function in some genetic backgrounds. Thus, confirming the function of each *Sr* gene is necessary after incorporation into a Sr-gene stack. The use of pathogen isolates is not possible due to the recognition of multiple avirulence (Avr) effectors that are delivered simultaneously. Transient expression of single *Avr* effectors can circumvent this limitation. However, this is currently limited by the scarcity of cloned *Pgt Avrs.*

I outline a procedure that could be used to accelerate *Avr* gene cloning through the development of a mutant population of *Pgt* spores followed by selection for gain-of-virulence mutants. To achieve this, I used ethyl methanesulphonate (EMS) of asexual haploid urediniospores and created a library of > 10,000 independent mutant isolates that were combined into 16 bulks of ~658 pustules each. We sequenced random mutants and the average mutation density was determined to be 1 single nucleotide variant (SNV) per 258 kb. Based on this, predictions of the minimum number of independently derived gain-of-virulence mutants required to confidently identify a given *Avr* gene was determined to be three. We screened the mutant library against plants containing *Sr43*, *Sr44*, or *Sr45* and identified 9, 4 and 14 mutants with virulence towards *Sr43*, *Sr44* or *Sr45*, respectively. Only mutants identified on *Sr43* and *Sr45* maintained their virulence after re-inoculation. Therefore, this method can select for mutants virulent towards targeted resistance (R) genes.

The development of a mutant library from as little as 320 mg spores creates a resource that can be screened against multiple R genes without the need for multiple rounds of mutagenesis and wildtype spore bulking.

(295 Words)

3.2 Introduction

To invade and parasitize plants, many pests and pathogens, (including *Pgt*), deliver effector molecules into host cells. Some of these effectors function as suppressors of host defenses by inhibiting immune response-signaling following pathogen recognition. Other effectors can regulate host gene expression (Ahmed et al. 2018) or function in nutrient acquisition during rust infection (Sohn et al. 2000; Thara et al. 2003). A subset of these effectors can be detected by the products of host resistance (*R*) genes encoding either extracellular or intracellular immune receptors, resulting in effector triggered immunity (ETI), which is usually associated with a visible hypersensitive cell death response (Peter N. Dodds and Rathjen 2010; Lo Presti et al. 2015a). These effectors are termed avirulence (Avr) effectors.

Cultivation of varieties carrying a single *R* gene in stem rust prone areas applies huge selection pressure which results in the appearance of virulent isolates of the pathogen (Johnson 1961; Vale et al. 2001; Hovmøller and Justesen 2007). However, well-planned stacking of multiple *R* genes in cultivars could, possibly increase the durability of resistance, as there would be a fitness cost to the pathogen associated with overcoming *R* genes in the stack (Huang et al. 2006; Zhang et al. 2009; Fukuoka et al. 2015).

Generally, for a given *R* gene in the host plant there is a corresponding *Avr* gene in the pathogen (Wulff and Moscou 2014). As such, the testing of each *R* gene in such stacks requires the identification of its corresponding Avr effector (Vleeshouwers and Oliver 2014; Wulff and Moscou 2014). *R* genes can negatively interact with each other when combined (Hurni et al. 2014), do not work in certain backgrounds (Hiebert et al. 2020), or can be silenced when introduced as transgenes (Anand et al. 2003; Li et al. 2005). This makes testing the individual function of each *R* gene in a stack necessary. Pathogens typically secrete multiple effectors

during infection and thus cannot always be used to test the function of single genes in the stack (Vleeshouwers and Oliver 2014; Wulff and Moscou 2014; Salcedo et al. 2017; Chen et al. 2017). Consequently, Avr effectors can serve as tools to test *R* gene stacks without the pathogen (Vleeshouwers and Oliver 2014; Upadhyaya et al. 2014; Bouton et al. 2018; Saur et al. 2019). In other studies, effectors have been used to study *R* gene structure/function relationships (Wulff et al. 2009; Maqbool et al. 2015; Ortiz et al. 2017; Seto et al. 2017a) as well as in the engineering of *R* genes with novel effector specificities (Harris et al. 2013; Segretin et al. 2014; Kim et al. 2016; De La Concepcion et al. 2019).

Several major dominant *Sr* genes have been cloned such as *Sr13* (Zhang et al. 2017), *Sr21* (Chen et al. 2018), *Sr22* (Steuernagel et al. 2016), *Sr33* (Periyannan et al. 2013a), *Sr35* (Saintenac et al. 2013a), *Sr45* (Steuernagel et al. 2016), *Sr46* (Arora et al. 2019), *Sr50* (Mago et al. 2015), *Sr60* (Shisheng Chen et al. 2020) and *SrTA1662* (Arora et al. 2019). Many more are underway. This makes the engineering of multi-gene stacks a possibility. Currently, there are only three cloned *Pgt Avr* genes namely *AvrSr27, AvrSr35 and AvrSr50* (Salcedo et al. 2017; Chen et al. 2017; Figueroa et al. 2020). Most wheat rust (*Puccinia sp.*) effector gene cloning strategies in *Pst* have used genome sequencing followed by bioinformatic effector prediction to shortlist candidate genes based on presence of a signal peptide, lack of homology to known proteins, absence of a transmembrane domain, being cysteine-rich, highly expressed during infection and being small in size (<300 amino acids) (Dagvadorj et al. 2017; Cheng et al. 2017; Zhao et al. 2018). However, no *Pst Avr* have been cloned as yet. Effector prediction strategies have also been largely successful for pathogens such as *Blumeria graminis* (Pedersen et al. 2012; Ahmed et al. 2015) *Phytophthora* sp*.* (Vleeshouwers et al. 2008)*, Bremia lactucae* (Stassen et al. 2012) and *Magnaporthe oryzae* (Chen et al. 2013). Effector identification is then followed by transient heterologous expression assays to test function of shortlisted candidates (Anderson et al. 2016). Despite multiple candidate rust effectors being listed, there are not yet any reliable high throughput mechanisms to test these effector candidates via transient expression in the cereal host. In the well-studied *M. lini* and *Bgt*, *Avrs* have also been cloned by methods such as map based cloning (Dodds et al. 2004; Praz et al. 2017). Furthermore, conventional bi-parental genetics and positional cloning is impractical due to the difficulties of performing sexual crosses under controlled conditions followed by handling large numbers of segregating progeny (Johnson 1954).

Mutagenesis and sequence-comparison of multiple independently-derived mutants can circumvent these challenges and is a promising approach which has been applied in the identification of genes from a wide array of organisms including worms, flies and plants (Wang et al. 2010; Ashelford et al. 2011; Steuernagel et al. 2016; Addo-Quaye et al. 2017; Kawamura and Maruyama 2019). EMS mutagenesis has also been successfully used to induce desirable functions in various microorganisms. The cotton pathogen *Ashbya gossypii* exhibited a 2-fold increase in the secretion of heterologously expressed endoglucanase I (EGI) and native α-amylase (Ribeiro et al. 2013). A total of seven mutants of the bacterium *Serratia marcescens* GPS-5 –used in biocontrol of groundnut leaf spot–showed increased ability to solubilize phosphorous, a trait which could find a use in improving availability of fixed soil P (Tripura et al. 2007). Other examples include the study of penicillin biosynthesis by *Penicillium chrysogenum*, enhanced lipid content in algae (*Nannochloropsis* sp.), increased uptake of atmospheric CO_2 and production of ethanol by *Clostridium carboxidivorans* (P7) (Masurekar et al. 1972; Doan and Obbard 2012; Lakhssassi et al. 2020).

Other mutagenesis approaches that have been tried in rusts include T-DNA insertion and irradiation. Disruption of the cloned *M. lini* AvrL567-A was accomplished via T-DNA insertion (Lawrence et al. 2010). The T-DNA vector (siAvrL567) expressed a silencing 480-bp hairpin RNA. In the case of *Pst*, X-ray treatment of pustules resulted in seven mutants with added virulence not present in the parental isolates (Stubbs 1968). Some of these, had virulence on *Yr5* (Stubbs 1968). UV radiation has been used for *Pst* mutagenesis resulting in gain-of-virulence to multiple *Yr* genes. This could be a result of UV mutagenesis causing single and double DNA strand breaks in addition to point mutations (Ikehata and Ono 2011). These DAN breaks can cause in loss of DNA segments resulting in loss of multiple effectors making the analysis of such mutants difficult to identify an *Avr* of interest since effector genes can occur in clusters.

EMS mutagenesis of *Pgt*, and *P. graminis* f. sp. *avenae* in the 1960s and 1970s, revealed the possibility of mutagenizing spores and selecting mutants with virulence towards defined resistance genes thus indicating loss of the function of the corresponding *Avr* genes (Teo and Baker 1966; Luig 1978). These historical and recent studies, however, did not provide

sufficient detail to enable reproduction of their mutagenesis procedures without first optimizing them. Recently, the EMS mutagenesis of the *Pst* isolate 11-281, which is avirulent on all 17 *Yr* genes in the stripe rust differential set resulted in the development of 33 virulent isolates that were characterized as 19 new and five existing *Pst* races (Y. Li et al. 2019). Sequencing and analysis of these isolates however did not result in the identification of any clear *Avr* candidates (Li et al. 2020). Since then, the development of genome sequencing technologies allowed the identification of the first *Pgt Avr* effectors (*AvrSr27*, *AvrSr35* and *AvrSr50*) which were cloned by comparing induced or natural mutants to their wild-type parents (Salcedo et al. 2017; Chen et al. 2017; Figueroa, et al. 2020). Specifically, *AvrSr35* was cloned by the use of EMS mutagenesis and sequence comparison of multiple independently derived gain-of-virulence mutants identified on wheat plants carrying *Sr35* (Salcedo et al. 2017).

The recognition of more than one *Avr* can hamper the success of Avr knockout using a random mutagenesis method such as EMS. In the cloning of *AvrSr35*, analysis to show its distribution among a diverse collection of 34 Pgt samples showed that there were five *Sr35*-avirulent isolates with at least two copies of the gene (Salcedo et al. 2017). The isolate used in EMS mutagenesis experiments to achieve gain-of-virulence against *Sr35* carried a functional copy of *AvrSr35* and an allele with the miniature inverted transposable element (MITE) in exon 6, which resulted in a premature stop codon which was predicted to produce a non-functional protein (Salcedo et al. 2017). This suggests that the presence of more than one allele or additional copies of the *Avr* recognised by an *Sr* gene of interest would significantly reduce the probability of knocking out two or more functional alleles to achieve a loss of recognition. Thus, the results can be influenced by the isolate used in the experiments.

In this present chapter, I optimized steps for creating EMS mutant libraries of *Pgt* using the isolate UK-01, race TKTTF by using ethyl methanesulfonate (EMS) (**Figure 1**). I present a detailed protocol on performing the mutagenesis, screening for virulent mutants towards defined target *Sr* genes, purification of the mutants, validation of their specificity, and increasing spore quantities of purified mutant isolates for sequencing. The screens conducted

against three important genes, *Sr43*, *Sr44* and *Sr45* in wheat provide proof of the effectiveness of this approach. A draft genome assembly of wildtype *Pgt* UK-01 was generated to improve precision in identification of EMS-induced mutations in the *Pgt* UK-01 genome without having to first mask isolate-specific SNPs when using other *Pgt* genome assemblies. I then conducted whole genome resequencing of eight randomly selected EMS-derived single pustule isolates of mutants from a single EMS experiment to calculate mutation density. These data enabled theoretical predictions of the minimum number of independently derived gain-of-virulence virulence mutants that would be required for resequencing to reduce the false positive rate in *Avr* gene identification to zero.

3.3 Results

3.3.1 Development of the mutagenesis procedure

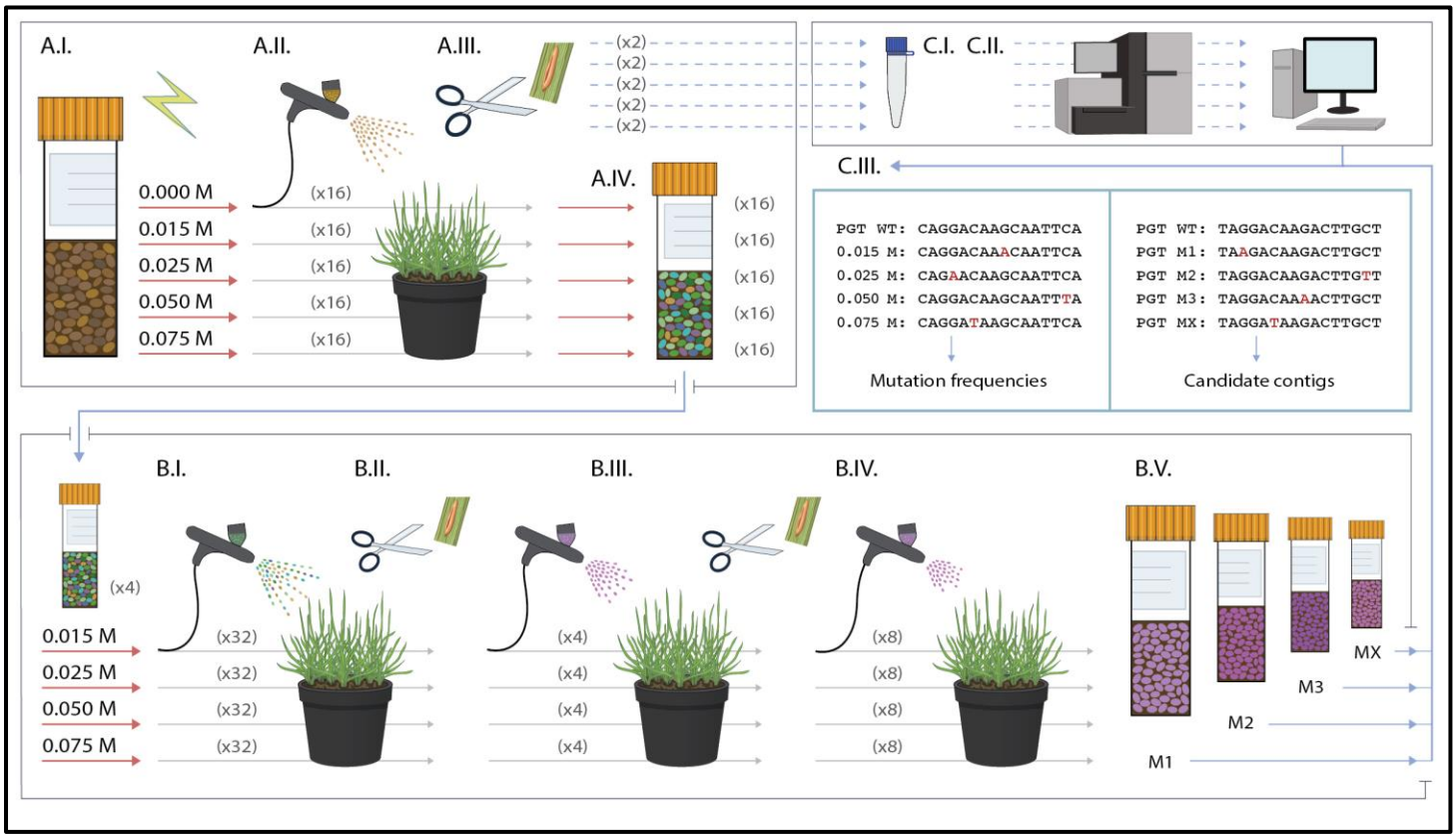


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Figure 3.1 | Overview of the development, screening, and analysis of *Pgt* EMS mutants.

Development of a *Pgt* mutant library starting with mutagenesis of the wildtype (A.i), inoculation of 16 pots each containing seven susceptible wheat plants per EMS treatment (A.ii), selection of two single pustule samples per treatment for spore increase, DNA extraction and sequencing (A.iii), followed by spore harvesting from each separate pot resulting in 16 mutant spore bulks per treatment (A.iv). The 16 bulks were pooled to make four master bulks per EMS treatment (B.i) and each bulk was inoculated on eight pots each containing seven plants of the *Sr* line of interest along with the *Pgt* wildtype control. Virulent pustules were isolated (B.ii), reinoculated onto the *Sr* line they were identified from (B.iii), and pure, single pustules were isolated for bulking up for downstream experiments (B.iv) and sequencing (B.v). Sample pustules from each EMS treatment were sequenced to determine SNV rates, whilst virulent mutants can be sequenced and analyzed to identify *Avr* candidate genes (C.iii).

In Chapter 2, I identified eleven *Sr* gene lines that exhibited sufficient resistance against the wildtype *Pgt* isolate utilized to allow detection of gain-of-virulence mutant *Pgt* pustules. I then proceeded to develop mutant libraries of *Pgt* isolate UK-01 (race TKTTF) in two mutagenesis experiments by incubating urediniospores in 0 M, 0.015 M, 0.025 M, 0.05 M and 0.075 M EMS solutions (Fig 3.1 A.I). These concentrations followed the mutagenesis protocol that was provided by the Steffenson group from their work on EMS mutagenesis of Ug99 to clone *Avr*. This range of EMS concentrations was developed around those that were used by Luig, Teo and Baker (1966, 1978). Luig (1978) used a single dose, 0.012 M EMS for *Pgt* and an incubation time of 2 h before inoculation. Teo and Baker (1966) used a dosage range, including 0.005 M, 0.01 M, 0.0125 M and 0.025 M for 2 h. To determine the appropriate incubation time for *Pgt* urediniospores, I observed the germination of untreated spores on water agar and determined that the initial stage of germination, i.e. tube emergence out of the spores, occurred at 1 h 40 min. I used this as the incubation time to minimize damage to protruding germtube due to the routine shaking during incubation in EMS followed by rinsing off and inoculation using an airbrush.

In my first attempt to create a *Pgt* mutant library outlined in Figure 3.1A, i – iv, I used the previously mentioned EMS concentrations and incubation time. I used 9 mg of spores per EMS treatment. Following rinsing with water, I inoculated 21-day old seedlings of a Triticale cultivar Rongcoo, which is resistant to leaf rust but is susceptible to stem rust. The resultant number of mutant pustules at 14 DPI was 52 for the whole experiment. Pustule count per treatment was: 0.015 M (25), for 0.025 M (13), 0.05 M (9), 0.075 M (3) (Fig 3.2 A, Annexe 2A). Analysis of pustule count per EMS concentration in EMS experiment 1 did not show a clear trend in pustule survival as the EMS concentration increased (Figure 3.2 A). The pustule counts for the treatments were not significantly different from the control ($P > 0.05$, Annexe 2B).

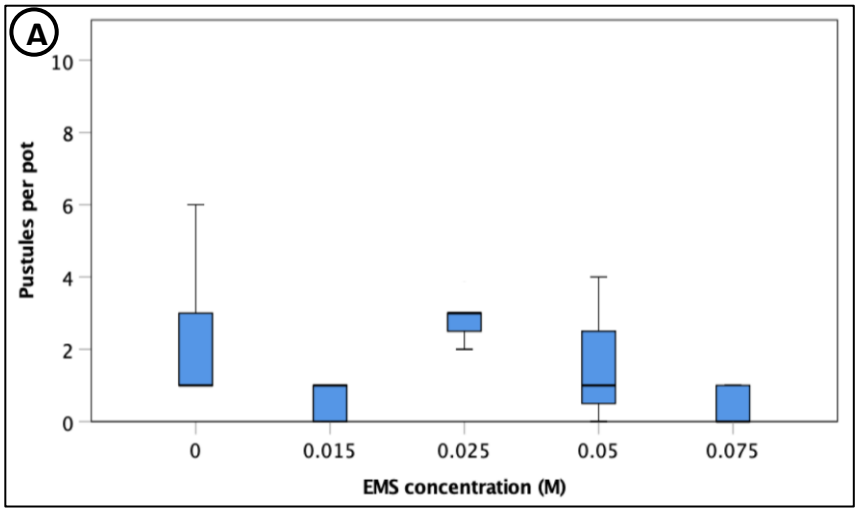
The low count of putative EMS-derived Pgt mutants obtained on Rongcoo , i.e. 49 pustules on a total of 224 plants (Annexe 2), in the whole experiment necessitated a redesign of the experimental procedure. The Triticale cultivar Rongcoo was initially used for inoculations because of its resistance toleaf rust, and its susceptibility to stem rust. However, its leaf width was narrower than that of the susceptible wheat cultivars in the lab stocks, but whose seed

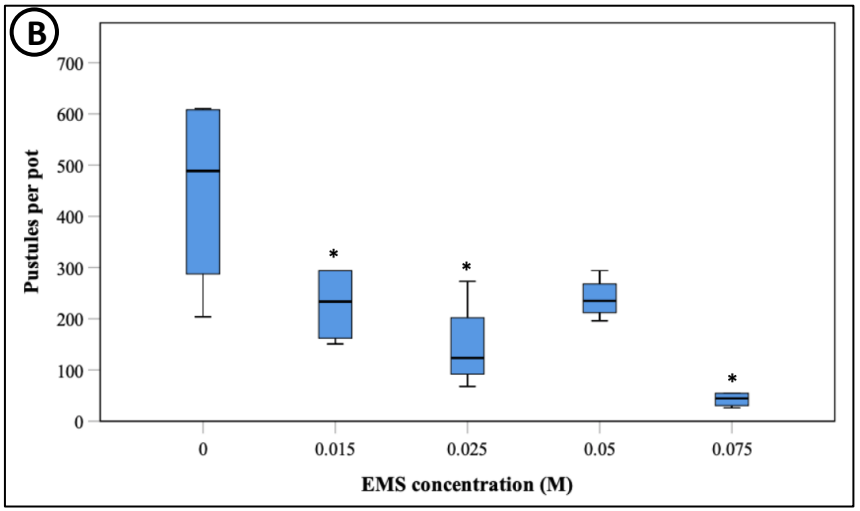
was still being multiplied for use in future experiments. The low amount of starting inoculum also contributed to low mutant recovery rates. Some mutations would be expected to be deleterious thus reducing the number of viable pustules. In addition, my modification of the EMS mutagenesis procedure by first bulking up mutant spores on a susceptible host (compared to directly inoculating a resistant plant as done by previous researchers) meant that only the fittest survivors would be included for the selection of gain-of-virulence mutants. The mutant inoculum was delivered using an airbrush onto plants with narrow leaves.

From these results, I conducted two further mutagenesis experiments adjusting the quantities starting spore material per treatment to 40 mg per EMS treatment to cater for EMS induced mortality and resorted to the use of younger seedlings of a highly susceptible wheat cultivar to enable better infection rates by the mutagenized spores. I then inoculated these mutagenized spores onto 128, 12 to 14-day old seedlings of the susceptible host cv. Chinese Spring per EMS treatment (Figure 3.1A, i – iv). To determine the effect of EMS mutagenesis on spore survival, I counted the number of leaf pustules from four randomly selected pots containing seven seedlings each for each EMS treatment as well as the water control at 13 days post inoculation (dpi) (Annexe 3 and 4). Analysis of pustule count per EMS concentration in EMS experiments 2 and 3 showed a clear decline in pustule survival as the EMS concentration increased (Figure 3.2). In EMS experiment 2, pustule counts for 0.015 M, 0.025 M and 0.075 M were significantly different (Fisher LSD) from the control whilst all EMS treatments in EMS experiment 3 were significantly different from the control.

Five weeks post inoculation, I harvested the resultant spores from each pot separately. In each of the two EMS experiments, sixteen pots were harvested per EMS concentration resulting in a total of 128 mutant collections (2 EMS experiments x 4 EMS concentrations x 16 pots = 128 harvests). The 16 collections in each EMS concentration were then combined into four bulks. As a result, I had two sets of 16 bulks. Based on the pustule counts from the sample pots at 14 dpi, I extrapolated the data to the whole experiment and estimate that I obtained 10,520 and 2,496 mutant pustules in mutagenesis experiments 1 and 2, respectively (**Annexe 2 and 3**). With this mutant population consisting of approximately 13,016 pustules, I

proceeded to the screening against selected *Sr* genes to identify gain-of-virulence mutants (Figure 3.1 B i – ii).





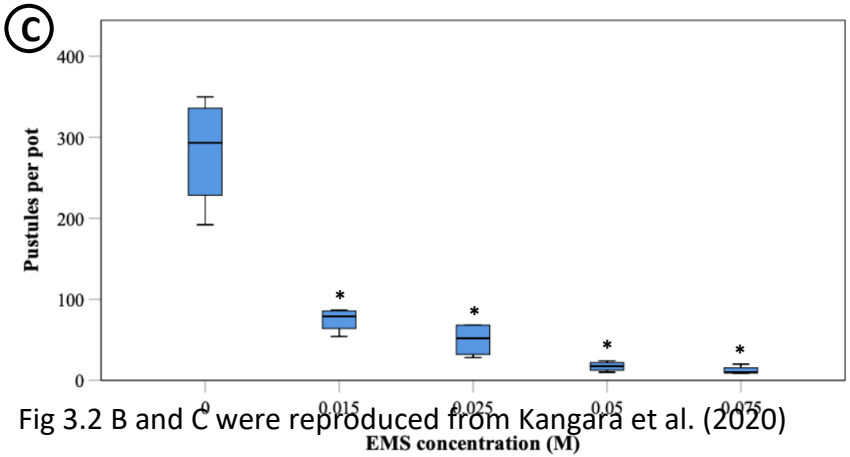


Fig 3.2 B and C were reproduced from Kangara et al. (2020)

Figure 3.2 | Pustule survival as an effect of different concentrations of the chemical mutagen ethyl methanesulphonate. Spores of Pgt UK-01 were subjected to different concentrations of EMS in mutagenesis experiments one (**A**), two (**B**) and three (**C**) and then inoculated onto leaves of 12-day old seedlings of triticale cultivar Rongcoo susceptible to Pgt (**A**) and seedlings of the susceptible wheat cultivar Chinese Spring (**B** and **C**). For each EMS treatment, 4 out of 16 pots were picked at random. Each pot contained seven plants. Pustules were counted 14 days post inoculation. The results from the three independent experiments are displayed in (A, B and C). EMS effect on pustule count in experiment one was not statistically significant (P>0.05). Asterisks indicate significant differences between treatments and null control according to Fisher's LSD test (B, P<0.01; C, P<0.01) (Annexes 2 - 6)

3.3.2 Generation of the wildtype *Pgt* UK-01 reference genome

My first step in creating a genome assembly of the wildtype *Pgt* UK-01 isolate involved extracting high molecular weight genomic DNA from urediniospores. Due to the difficulty in attaining high yields of good quality high molecular weight DNA (HMW-DNA), I tested out published protocols before modifying one to improve DNA quantity and quality. Initially, my DNA yield was around 300 ng from 100 mg of ureidiniospores. Increasing the starting spore quantity for DNA extraction did not correlate with a substantial increase in DNA yield, so I abandoned this as a potential avenue for obtaining more DNA, given the challenges of obtaining large amounts of spores. As part of a wheat rust genome (*Pgt*, *Pst*, *Pt*) sequencing project within the Saunders group, a decision was made to use 10X Chromium sequencing provided by the Earlham Institute (EI), Norwich, which requires low quantities of HMW-DNA. Working with Jessica Meades from the Saunders group, I used a complex three-day protocol involving embedding released nuclei from crushed spores in agar followed by lysis steps to release the DNA within the agar plugs to prevent shearing (Zhang et al. 2012). However, the DNA did not meet the minimum quality control requirements at EI. Q-Card and Femto-pulse readings showed the presence of HMW-DNA fragments with the largest being 163 kb (**Annexe 7**). These were not in adequate amounts compared to most smaller fragments. After trying several times, I resorted to using a protocol designed for obtaining DNA suitable for MinION sequencing (Nagar and Schwessinger 2018). The first attempt yielded 150 ng of high-quality DNA. With subsequent reduction in chloroform:isoamyl alcohol (24:1) (C:I) extraction steps and precipitation of DNA with isopropanol in place of CTAB DNA yields increased to 1.2 µg. This DNA was sent to Novogene for 450 bp insert size PCR free library preparation and 250 bp paired-end sequencing with the aim of generating a *de novo* wildtype assembly. However, the library preparation failed.

Nicola Cook of the Saunders group, who then performed long-read sequencing using the Oxford Nanopore Technologies' (ONT) MinION platform. ONT MinION is a portable, pocket-sized device which uses nanopore sequencing technology. It uses direct, concurrent examination of long DNA or RNA fragments by identifying alterations in the ion current of nucleotide sequences of a DNA strand as it passes through a membrane-inserted protein

nanopore. This data is decoded to provide the specific DNA or RNA sequence. This enables analysis of native DNA and does not require amplification which other DNA sequencing technologies such as Illumina rely on thus eliminating PCR bias. It also enables resequencing of long DNA fragments which is advantageous in genome assembly. However, its major drawback is the high error rate of 12 to 35 % (Lu et al. 2016).

Using MinION Nanopore sequencing, Guru generated 5.22 Gb of raw reads which are equivalent to an estimated 30-fold coverage of the ~170 Mb dikaryotic *Pgt* genome (Li et al. 2019). The average read length was 2.58 kb, while the minimum and maximum read lengths were 0.08 and 51 kb, respectively (**Table 3.1**). Reads longer than 1 kb were selected for use in the genome assembly. Dr Radhakrishnan first assembled the MinION reads with Canu and obtained an assembly size of 163.4 Mb with 4,902 contigs and an N50 of 53.3 kb (**Table 3.1**).

Following this, I obtained a total of 19.7 Gb of Illumina short read data from one 450 bp insert library with 250 bp paired end reads with Novogene, China. I shared the Illumina data and the Nanopore assembly of *Pgt* UK-01 with our collaborators Tomasz Kurowski and Fady Mohareb, Cranfield University, UK.

Finally, due to the high error rate of Nanopore MinION sequencing (Laver et al. 2015), Kurowski conducted error correction using the Illumina data to improve the genome assembly accuracy to allow for better precision in SNV calling. Following polishing the final assembly size was 164.3 Mb (**Table 3.1**). It can therefore be estimated that we assembled 96.6% of the ~170 Mb dikaryotic *Pgt* genome. Assessment of genome completeness revealed that the Nanopore-only assembly contained over 76.7% of conserved fungal BUSCO genes which improved to 93% after polishing (**Table 3.1**).

Table 3.1 | ***Pgt* UK-01 wildtype assembly statistics**.

Parameters	Nanopore assembly	Hybrid assembly
No. of contigs	4,902	4,902
Contigs ? 10,000 bp	3,956	3964
Total length (Mb)	163.4	164.3
Total length ? 10,000 bp	158.2	159.1
N50 (kb)	56.2	53.6
Min (kb)	1.01	1.01
Max (kb)	570.8	572.4
L50	812	812
GC (%)	43.45	43.45
% complete BUSCOs	76.7	93.9
% single-copy BUSCOs	50.6	47.3
% duplicated BUSCOs	26.1	46.6
% fragmented BUSCOs	16.4	3.6
% missing BUSCOs	6.9	2.5

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3.3.3 Density of EMS-induced mutations in the *Pgt* genome

To establish the genome-wide mutation density I first collected nine random pustules from the first EMS population. After bulking up, I collected enough spores for 150 bp paired end sequencing of one to two pustules per EMS treatment (**Figure 3.1A**, iii). Tomasz Kuroswki aligned the raw reads for each of the mutants to the final wild type genome assembly and called single nucleotide variants (SNVs). To limit the discovery of false G:C to A:T transitions, we asked Tomasz Kuroswki to use the following filters:

(i) Consideration of only G:C to A:T transition mutations which typically account for around 98% of EMS-induced mutations (Lebkowski et al. 1986; Krasileva et al. 2017).

(ii) Removal of SNVs which were common between the mutant samples and 0 M control.

(iii) Removal of SNVs that were present in three or more mutants.

The number of SNVs called by GATK (McKenna et al. 2010) per mutant genome ranged from 1,490 to 2,289, reflecting a G/C to A/T transition density per treatment of 1 per 103 kb for

0.015 M, 1 per 97 kb for 0.025 M, 1 per 87 kb for 0.05 M, and 1 per 80 kb for 0.075 M and were nearly similar to those called by SAMtools (Li et al. 2009) (**Table 3.2**). However, the SNV overlap between the two pipelines resulted in a SNV density of 1 per 404 kb for 0.015 M, 1 per 393 kb for 0.025 M, 1 per 239 kb for 0.05 M and 1 per 182 kb for 0.075 M. This amounted to an estimated total of 5,982,736 high confidence SNVs in the population created in EMS experiment 2 that could be screened against *Sr* genes of interest (Table 3.3). Thus, each of the four bulks per EMS treatment would contain on average 399,000 SNVs for 0.015 M, 254,604 SNVs for 0.025 M, 681,600 SNVs for 0.05 M and 160,480 SNVs for 0.075 M (Table 3.3). The SNV densities were positively correlated with the EMS concentration ($r^2 = 0.9$, in the case of the high confidence SNV densities) (**Annexe 8**). The density of total, non-redundant SNVs per treatment ranged from 1 SNV per 56 kb to 1 SNV per 44 kb.

From a total 71,388,350 G/C pairs in the assembly (Table 3.1), the average total non-redundant G/C to A/T transitions of 3,287 SNVs (Table 3.2) showed that the amount of G/C to A/C transitions were 0.005% per mutant. This was lower than the observations made in the EMS mutagenesis in wheat which was 0.0243% (Sánchez-Martín et al. 2016). However, in the same study, a lower frequency of 0.00928% was observed in Barley. This suggests a lower tolerance for deleterious mutations with decrease in organism ploidy (Tsai et al. 2013; Sánchez-Martín et al. 2016; Uauy et al. 2017). With roughly comparable contig lengths for Barley (12kb) and Wheat, (10kb) the number of mutants required to resolve the candidate contigs (gene) mutated in multiple individuals was four and three respectively (Sánchez-Martín et al. 2016). For *Pgt* UK-01, it is expected that longer contigs might reduce the number of independent mutants required to find the candidate contig with high confidence.

Table 3.2 | **Mutation frequency per EMS treatment***.

		SNV count				Minimum no. of mutants required to identify a gene by mutational genomics	
Sample[a]	**EMS level (M)[b]**	**GATK**	**Samtools mpileup**	**High confidence SNV[c]**	**High + low confidence[d]**	**Based on high confidence SNVs[e]**	**Based on low confidence SNVs[f]**
				GATK + Samtools shared	Non-redundant SNVs		
B1	0.015	1490	1893	351	3032	3	5
B2	0.015	1678	2358	524	3512	3	5
C2	0.025	1680	1920	433	3167	3	5
D1	0.05	1861	2307	701	3467	3	5
D2	0.05	1894	1889	719	3064	3	5
E1	0.075	2289	2599	1033	3855	4	5
E3	0.075	1829	1939	855	2913	3	5

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*Please refer to the notes after table 3.2 explaining the column headings.

Table legend:

a. ID of single pustules collected for sequencing for each EMS treatment
b. EMS concentrations used to mutagenize *Pgt* UK-01
c. Similar SNVs that were identified by both tools.
d. The total number of unique SNVs (both high and low confidence) called by GATK and Samtools.
e. Prediction of the minimum number of virulent mutants required to identify the *Avr* using high confidence SNVs.
f. Prediction of the minimum number of virulent mutants required to identify the *Avr* using SNVs called by either GATK or Samtools (low confidence SNVs).

Table 3.3 | Estimated single nucleotide variation count per bulk for the EMS population that was generated in EMS experiment 1.

Bulk sample[a]	EMS level (M)[b]	High confidence SNV[c] GATK + Samtools shared	Experiment pustule Count per EMS level[d]	Total SNVs[e]	Av. SNV per EMS level[f]	Av. per bulk[g]
B1	0.015	351	3648	1280448	1596000	399000
B2	0.015	524		1911552		
C2	0.025	433	2352	1018416	1018416	254604
D1	0.05	701	3840	2691840	2726400	681600
D2	0.05	719		2760960		
E1	0.075	1033	680	702440	641920	160480
E3	0.075	855		581400		
	Total	4616	**6872**	**10947056**	**5982736**	**1495684**

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*Please refer to the notes below explaining the column headings of Table 3.3.

Table legend:

a. ID of single pustules collected for sequencing for each EMS treatment
b. EMS concentrations used to mutagenize *Pgt* UK-01
c. The (same) SNVs that were identified by both tools.
d. The total number of pustules that were counted on plans inoculated by spores for each EMS treatment.
e. The number of high confidence SNVs per sample multiplied by the number of pustules per EMS treatment.
f. The sum of the total SNVs for the samples per treatment divided by the number of samples
g. There were 16 pots inoculated per EMS treatment. The spores from each pot were collected separately and then pooled into four bulks per EMS treatment. The average SNV per bulk was calculated by dividing the number of mutant bulks (4) per treatment.

The cloning of high confidence *Avr* candidate genes by mutational genomics requires multiple independently derived gain-of-virulence candidates to resolve the region with the causative mutations resulting in the common phenotype upon sequence alignment. Sreya Ghosh in the Wulff group at the JIC calculated the minimum number of independently derived gain-of-virulence *Pgt* mutants required considering the following criteria:

(i) Sliding windows that were 5 kb for contigs larger than 5 kb since on average there is 1 gene per 5 kb in *Pgt* (Duplessis 2011; Li et al. 2019).

(ii) An observed mean GC content of 42.9% (with a standard deviation of 3.8%) per contig/window in *Pgt* UK-01 (**Annexe 9**).

(iii) The canonical-EMS SNV density (**Table 3.2**).

Simulations for the total number of false positives (i.e. 1-5 kb windows with mutations in all compared mutants by chance alone) were then calculated for analysis of 1 up to 10 mutants according to the principle of a binomial distribution. The minimum number of independently derived *Pgt* mutants required to reduce the probability of calling false positives to zero was three across all EMS treatments (**Table 3.2**) when considering the high confidence SNVs. This number increased to five mutants when using the complete set of non-redundant SNVs (**Table 3.2**). Therefore, the higher minimum as determined using low confidence SNVs sets the minimum target number of mutants virulent to towards an *R* gene that need to be identified during *Pgt* mutant screens.

3.3.4 EMS mutagenesis of *Pgt* UK-01 allows recovery of mutants with stable virulence to *Sr43* and *Sr45*, but not *Sr44.*

The 13,016 independent pustules from *Pgt* mutant populations I generated in mutagenesis experiments two and three with 8,583,122 unique, high confidence mutations gave me the assurance to start my screens on shortlisted *Sr* genes starting with the wheat-*Th. intermedium Sr44* introgression line (IT ;). I screened 75% of the mutants on *Sr44* and at 21 dpi, I identified four weakly virulent mutant pustules which had an IT of 1+ (**Figure 3.3-1**, mutants M-1 and M-2; **Annexe 10**). Some pustules (two) were located close to powdery mildew pustules that had contaminated the experiment. These pustules required purification and bulking up before use in experiments to confirm their phenotype. Following re-inoculation on *Sr44* the mutants did not maintain their virulence and reverted to wildtype IT ;.

I proceeded to screen the mutant libraries from mutagenesis experiments two and three containing a total of 13,016 *Pgt* mutants, on the Wheat-*Th. ponticum Sr43* line. From this screen, I obtained twelve gain-of-virulence mutants at 16 dpi (**Figure 3.3-2**). All virulent mutants had an IT of 3 or 4 whilst the IT of the wildtype control, *Pgt* isolate UK-01 on the *Sr43*

line was 1+ (**Figure 3.3-2**, **Annexe 10**).. Infection types of the mutants on *Sr43* were 3 for M-1, M-4, M-5, M-7, and M-10 whilst mutants M-3, M-6, M-8, M-9, M-11, M-12 and M-13 has size 4 pustules (**Fig 3.3-2, Annexe 10**). These mutant pustule sizes became distinguishable from those of the wildtype at 12 dpi and reached full size at 16 dpi I reinoculated the mutant pustules back onto *Sr43* to confirm their phenotype. During this, I observed that four mutants exhibited variation in their phenotypes. For each mutant, I isolated the pustules with the best phenotype and reinoculated *Sr43* to purify the isolate and confirm maintenance of virulence. A total of nine out of twelve mutants maintained their phenotype. During the multiplication of purified pustules, I also observed leaf rust and powdery mildew contamination which would have affected mutant sequence analysis. However, I was able to recover the virulent mutants via single pustule isolation.

Finally, I screened the 13,016 mutant pustules against *Sr45* in the wheat-*Ae. tauschii Sr45* stock, or the *Ae. tauschii* accession TOWWC191 which is predicted to only contain *Sr45*. From this screen, I identified fourteen virulent mutants with IT 2+ (M-3, M-9, M-11), IT 3 (M-1, M-2, M-8, M-12, M-13, M-13) and IT 4 (M-4, M-5, M-6, M-10) (**Figure 3.3-3**, **Annexe 10**). The *avrSr43* and *avrSr45* pustules had no apparent change in colour or morphology when inoculated onto the cv. Chinese Spring recurrent parent compared to that of the wildtype *Pgt* isolate UK-01. The main differences were in the slow rate of growth and low sporulation of some of the mutants (but not all) compared to the wildtype. The mutants identified on *Sr43* and *Sr45* were sent for Illumina sequencing at Novogene, China. Analysis of the mutant data is currently underway by the Mohareb group.

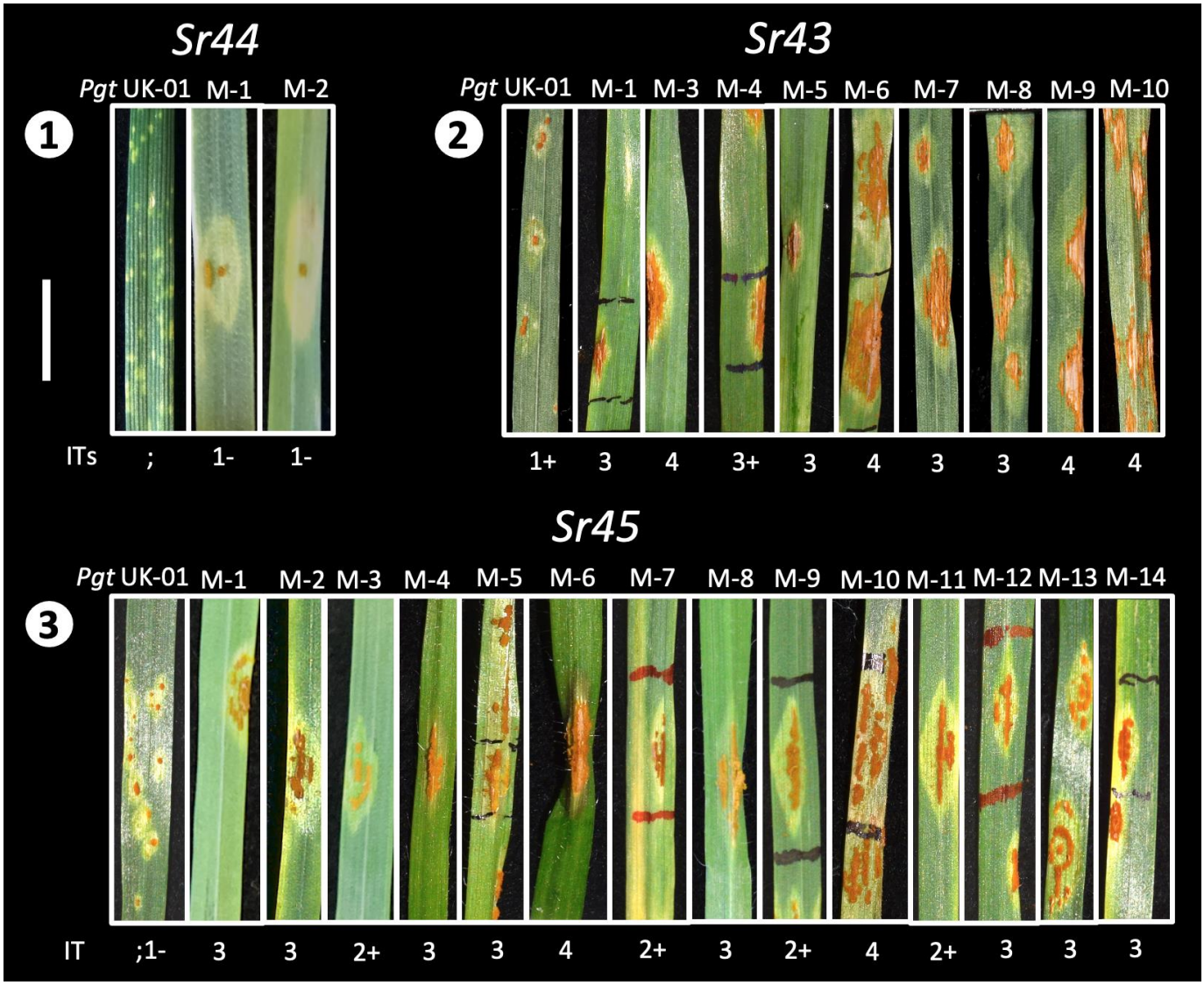

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Figure 3.3 | ***Pgt* UK-01 EMS mutants with virulence to *Sr44*, *Sr43* and *Sr45*.**

The wheat lines carrying *Sr44*, *Sr43* and *Sr45* together with their susceptible parent lines were inoculated with wildtype and mutant *Pgt* UK-01. Each of the 16 mutant bulks was screened on 56 resistant plants. Pustule infection type on the controls of wildtype inoculations on *Sr* lines were resistant (*Sr44* IT ;, *Sr43* IT 1+, *Sr45* IT 1). Virulent pustules were identified at 16 dpi. Pustule size was determined visually using the scale by Stakman et al., (1962). Each mutant within a series was obtained from different mutant bulks and they were only selected if the pustule size was significantly larger than that of the wildtype *Pgt* UK-01 control. The virulent pustules were isolated for purification and were reinoculated on the line that they were identified on to confirm maintenance of virulence. The isolate that was used in the inoculations is at the top of each sample image and the infection type is at the bottom. Bar represents 1 cm.

3.4 Discussion

Next generation sequencing and computational technologies have enabled the cloning of several *Sr* genes and many more are in the pipeline (Keller et al. 2018; Periyannan 2018). Thus, the engineering of multi-*Sr* gene stacks for durable stem rust resistance is a possibility. On the other hand, the cloning of their corresponding *Pgt Avr* genes has progressed slowly as they do not have defined sequence features making sequence analysis to identify *Avrs* difficult. To overcome this and improve the rate of *Pgt Avr* gene cloning, I have outlined a procedure for the generation of mutant populations of *Pgt* spores and selection of multiple independent gain-of-virulence mutants towards targeted *Sr* genes. Our work builds on studies from the '60s and '70s where chemical mutagenesis led to development of rust isolates virulent against defined *R* genes in oat and wheat (Teo and Baker 1966; Luig 1978). I innovated the development of bulks of mutant *Pgt* spores which creates a resource that enables screening against several *R* genes without the need for multiple rounds of spore bulking and mutagenesis.

In this chapter I present a detailed procedure for the creation of mutant *Pgt* libraries which can be returned to again and again as a resource for multiple mutant screens. My innovation was based on previous work in the 60s, 70s and more recently in 2017 (Teo and Baker 1966; Luig 1978; Salcedo et al. 2017). I introduced a step where I first inoculated mutagenized spores onto a susceptible cultivar, Chinese Spring, thereby multiplying individual mutant spores. The covering of individual pots with breathable bags containing the inoculated Chinese Spring plants prevented cross contamination once sporulation began and enabled harvesting of the spores from each pot. Creating a population of *Pgt* mutants meant multiple *Sr* lines could be screened in sequential experiments in a confined containment space. Also, this reduced the need to repeat mutagenesis for each screen and maximized the use of the limited starting urediniospore material of 400 mg of wildtype urediniospores (including the control) in both experiments.

In previous *Pgt* EMS mutagenesis studies spores were directly inoculated onto plants carrying defined *Sr* genes after mutagenesis (Teo and Baker 1966; Luig 1978; Salcedo et al. 2017). This requires a large amount of initial wildtype inoculum and generation of new mutants at the

start of any new screen. In my method, a mutant urediniospore library can be stored for long periods of time in a -80 ºC freezer or liquid nitrogen. Aliquots of the mutant bulks could be taken from storage and used as and when required. This strategy allowed me to select independently derived gain-of-virulence mutants on either *Sr43* or *Sr45* as only one virulent pustule was selected per mutant bulk. The harvested mutant spores from each pot of the 16 pots were kept separate per EMS treatment and were then pooled into batches of four per EMS treatment (to create 16 bulks per experiment). This is important as multiple independent mutants are required for analysis to identify the *Avrs* through causative mutations present in all or most individuals (Sánchez-Martín et al. 2016b).

Pustule counts during mutant library creation revealed a decline in pustule survival with increasing EMS concentration (**Figure 3.2**). This was associated with an increase in the density of high confidence SNVs per EMS treatment from 1 SNV per 484 kb at the lowest EMS concentration to 1 SNV per 165 kb at the highest concentration. At the population level there were an estimated total of 8,583,122 unique SNVs within the two populations. Thus, for the estimated *Pgt* genome size of 170 Mbp, this would be 1 SNV per 21 bp (**Table 3.2, Table 3.3**).

The increase in deleterious mutations with increasing mutation densities likely explains the sharp decline in pustule survival with increasing SNV density. The average mutation rate of 1 SNV per 258 kb for high confidence SNVs per individual mutant indicated that the EMS treatments were effective, and candidate *avr* loss of function mutants could be identified. In the previous *Pgt* mutagenesis screen which led to the cloning of the *AvrSr35* effector, the mutation density ranged from 1 SNV per 2,152 kb to 1 SNV per 10 kb with the average mutation rate being 1 SNV per 77 kb (Salcedo et al. 2017). Our observed mutation densities for the total unique SNVs called by both GATK and Samtools was slightly higher but fell within the previously observed range with an average SNV rate of 1 SNV per 52 kb. I identified that just a 1.3-fold increase in mutation density from lowest and highest EMS concentration resulted in a high (5 to 8-fold) decline in pustule count. However, there was no clear difference in the frequency of obtaining gain-of-virulence mutants between the different EMS concentrations, expressed as number of mutants obtained per 10^6 SNVs screened (**Table 3.2**), although the lowest EMS concentration did not yield any clear gain-of-virulence mutants.

Therefore, EMS treatments around 0.025 M EMS would be useful in *Pgt* mutant screens as they have more spore survival versus a minor decline in SNV density.

I proceeded to screen the mutant populations on *Sr43* and *Sr45*. I identified 11 virulent mutants on *Sr43* and 14 gain-of-virulence mutants on *Sr45*. This might indicate *Pgt* UK-01 could be heterozygous for *AvrSr43* and *AvrSr45*. However, I did not get any stable mutants for *Sr44*, suggesting that *Pgt* UK-01 may contain a copy of *AvrSr44* in both of its haploid nuclei making it unlikely to obtain loss-of-function mutants as both copies would have to be knocked out. Another explanation could be that the introgressed *Th. intermedium* segment carries another *Sr* gene in addition to *Sr44* that recognises another *Avr* in *Pgt* UK-01.

Using the average high confidence SNV rates within the *Pgt* genome, Sreya calculated that three independently derived mutants would be the minimum number of mutants required to clone a candidate *Avr* gene when considering high confidence SNVs. In the case of non-redundant SNVs, five mutants are the minimum number. These calculations are based on the assumption that there is a "gene-for-gene" *Avr-Sr* interaction (Flor 1955) where the change in *Pgt* phenotype from avirulence to virulence against an *Sr* line can be attributed to intragenic mutation(s) in the *Avr* as opposed to a second site suppressor (Figueroa et al. 2020). The appearance of the gain-of-virulence mutant pustules was similar to that of wildtype *Pgt* UK-01 on Chinese Spring which is the recurrent parent for the *Sr43* and *Sr45* introgression lines. However, Teo and Baker (1966) observed some EMS mutants of *P. graminis* f. sp. *avenae* which developed teliospores early on resistant lines, while (Luig 1978) reported *Pgt* pustules that were yellow or orange. Salcedo et al. (2017) did not report any such changes in *Pgt* mutants.

In Chapter 4, experiments to determine the *Pgt* race of the *Sr43* and *Sr45* gain-of-virulence mutants on the stem rust international differential set confirmed that the mutants retained the same TKTTF race designation of the wildtype. This indicated that the gain of virulence observed was likely due to loss of a single effector gene (*AvrSr43* or *AvrSr45*) rather than contamination from other isolates.

In summary, I have developed a protocol for creation of *Pgt* mutant populations and obtaining *Pgt* gain-of-virulence mutants. I successfully demonstrated this by obtaining gain-of-virulence mutants towards either *Sr43* or *Sr45.* This work, in combination with analysis of mutant whole genome sequence data (Salcedo et al. 2017) could enable the cloning of *AvrSr43* and *AvrSr45.* This method can be applied to knockout mutants in other *Avrs* of *Pgt* and other fungal pathogens with haploid genomes that can be propagated using asexual spores. Further downstream, I expect the near complete pan-genome complement of *Pgt Avrs* to be cloned. This will have applications in regional and global sequence-based monitoring of the virulence of *Pgt* populations in the field and allow breeders to judiciously deploy cultivars carrying effective *Sr* multi-gene stacks.

3.5 Materials and methods

3.5.1 Mutant library creation

Seedlings of the universally susceptible cultivar Chinese Spring were prepared by sowing sixteen pots of 12 to 14-day-old seedlings with seven wheat seeds per 9 x 9 cm pot. The seedlings were grown in a controlled environment room as described in Chapter 2. For EMS mutagenesis, 200 mg of *Pgt* UK-01 urediniospores were "heat shocked" in a water bath at 45 ^{o}C for 10 minutes. Solutions of 0 M, 0.015 M, 0.025 M, 0.05 M, 0.075 M EMS were prepared using sterile H_2O with 0.01% Tween 20 in 50 ml Greiner tubes. I added 40 mg of urediniospores to each tube and gently shook the suspensions by hand every 20 minutes until 1 hour and 20 minutes. The spore suspensions were separately filtered through Whatman cellulose filter paper by gravity filtration. The EMS solution was washed from the spores with 500 ml of sterile water with 0.01% Tween 20. After being drained, the urediniospores were transferred into 30 ml Nalgene^{TM} Oakridge tubes by washing off the filter paper using sterile water with 0.01% Tween 20. All equipment contaminated with EMS was immersed in EMS inactivation solution made up of 0.1 M NaOH and 10 % w/v $Na_2S_2O_3$ for at least 24 h in the fume hood.

The mutagenized spores suspended in water with 0.01 % Tween 20 were immediately used to inoculate cv. Chinese Spring plants in a Class 2 biological safety cabinet using an airbrush. The full 30 ml of inoculum per EMS treatment were used per set of 16 pots containing a total

of 112 plants (**Figure 1A** i - iv). Following inoculation, the procedures for misting and growth of the plants were described in Chapter 2.

To determine the EMS mutation rates, I randomly selected two single pustules per treatment at 12 dpi, for purification, bulking up and whole genome shotgun sequencing. To determine pustule survival per EMS treatment I selected four pots at random per EMS treatment and counted pustules on the leaves of all the plants in the pots at 14 dpi. The breathable cellophane bags on the pots for the mutant library creation as well as purified sample pustules were bent sideways to allow the spores to collect in the bags. At 35 dpi, the spores were collected from the cellophane bags. Spores were dried for four days over silica beads and stored at -80 ^{o}C for future use.

3.5.2 DNA extraction and whole genome shotgun sequencing

Sample pustules were harvested, diluted with Novec, and then inoculated onto four pots containing seedlings of cv. Vuka. The mono-pustule isolations were conducted twice. In the third cycle, 70 - 100 mg of urediniospores per sample were used for a CTAB DNA extraction. Urediniospores were mixed with 50 mg of glucose/sucrose mix and 50 mg sand in a mortar and pestle followed by grinding to a fine powder. Prewarmed CTAB buffer (50 ^{o}C) was added to the ground mix followed by 10 µl proteinase K (20 mg/ml) and incubation at 50 ^{o}C for 2 hours with intermittent shaking. Another 10 µl proteinase K (20 mg/ml) was added followed by incubation at 50 ^{o}C for 1 h. After this, 1 volume (V) chloroform:isoamyl alcohol (24:1) was added and the mix shaken vigorously and centrifuged at 5,000 g for 10 minutes. A total of 20 µL RNase (0.1 mg/mL) was then added with incubation at room temperature for 1 hour. A second chloroform:isoamyl extraction and centrifugation was then conducted. DNA was precipitated from the aqueous phase using 1 V chilled (-20 ^{o}C) isopropanol and left overnight in a -20 ^{o}C freezer. DNA was pelleted by centrifugation at 16,000 g for 10 minutes. The DNA pellets were washed twice with 1 ml of 70 % chilled ethanol and centrifuged at 16,000 g after each wash. The DNA pellets were dried, and then suspended in 70 µl 1 % TE buffer. DNA quantification and quality analysis were carried out using a Nanodrop^{TM} spectrophotometer and agarose gel electrophoresis and comparison to known concentrations of Lamdha phage DNA. Illumina 350 bp insert library preparation and 150 bp paired-end whole genome shotgun sequencing was conducted at Novogene, Beijing or Genewiz.

3.5.3 High molecular weight DNA extraction, MinION library preparation, sequencing, and assembly

I obtained High molecular weight gDNA from *Pgt* UK-01 using a protocol published by Nagar and Schwessinger (2018). The steps in the protocol were followed with the following adaptations: (i) the lysis buffer containing the ground spores was left to stand for 30 minutes before Proteinase K was added (ii) after the first chloroform:isoamyl alcohol (24:1) step, 0.1 V of 3 M sodium acetate (pH 5.2) was added to the aqueous phase and then DNA was precipitated by addition of chilled (-20 ^{o}C) 1 V isopropanol followed by pelleting at 16,000 g for 5 min, washing the DNA pellet with chilled 70% ethanol, drying and elution in 500 µl 10 mM Tris HCL, (iii) after RNase digestion for 1 hour, Proteinase K digestion was conducted according to the protocol before the second (final) chloroform:isoamyl alcohol (24:1) step followed by DNA precipitation using 1 V isopropanol, pelleting, ethanol washing, DNA pellet drying as previously described and elution in 200 µl TE buffer.

The DNA was then prepared for sequencing on the MinION sequencer (Oxford Nanopore Technologies, Oxford, UK). The DNA concentration was measured using a Qubit fluorometer (Thermo Fisher Scientific). A total of 305 ng of DNA was used as input for library preparation using the 1D Ligation Sequencing Kit (SQK-LSK109, Oxford Nanopore Technologies) carried out as per the manufacturer's instructions. The resulting library had a total mass of 67 ng and was sequenced on the MinION using a FLO-MIN106D flow cell (Oxford Nanopore Technologies), with 1,378 pores available for sequencing, following the manufacturer's instructions for a total of 48 hours.

Basecalling of the MinION reads was performed using Guppy v3.0.3 (https://community.nanoporetech.com) on CPU mode using the default parameters. Following basecalling, only reads longer than 1 kb were taken forward for genome assembly using Canu v1.8 (Koren et al. 2017) using the default parameters and an estimated genome size of 170 Mbp. Genome completeness was assessed using BUSCO v3. (Waterhouse et al. 2018) for the Basidiomycota fungal lineage on genome mode with *Ustilago maydis* as the reference species for gene prediction using Augustus v3.2.1 (Stanke and Morgenstern 2005).

3.5.4 Polishing of the wildtype long read assembly

To conduct error correction of the Nanopore draft assembly, I generated PCR Illumina data by sequencing 250 bp paired-end Illumina reads with average insert size of 450 bp. A total of 19.7 Gbp of data was obtained and aligned to the contigs of the Nanopore assembly using BWA-MEM 0.7.15 (Li and Durbin 2009). The alignment results were sorted and duplicates were marked using Picard 2.18 (Broad Institute 2009) as the data were generated from PCR libraries. The alignment data were then used to improve the draft assembly using Pilon 1.23 (Walker et al. 2014) with the diploid setting and the default fix list: attempting to correct individual base errors, indel errors and local misassemblies, as well as fill gaps. This was repeated five times until the number of changes applied to the draft assembly plateaued. The resulting polished genome was re-assessed using BUSCO v3 (**Table 3.1**) and used as the reference for downstream analysis.

3.5.5 Determining EMS mutation rates

Whole genome resequencing data from eight EMS-derived mutants consisting of 150 bp paired-end Illumina reads were aligned to the polished *Pgt* UK-01 genome assembly using BWA-MEM 0.7.15. As with other Illumina alignments, the results were sorted, and duplicates were marked using Picard 2.18. The GATK 3.8 (McKenna et al. 2010) IndelRealigner tool was then used to create the final BAM alignment files for each of the mutants.

Variant calling was then carried out using two different tools, GATK 3.8 HaplotypeCaller and bcftools 1.6 (Danecek et al. 2011), yielding two sets of results per sample. The results were filtered with a basic GATK hard filter, removing low-quality or low-depth calls with QD below 2 and MQ below 40. The entire pipeline has been carried out for each of the eight mutant samples separately.

Seven of the eight samples (B1, B2, C2, D1, D2, E1, E3) in both sets of variant calls were then passed through a filtering pipeline aimed at identifying EMS-induced SNVs. The eighth sample (A1) was the control, *i.e.,* not treated with EMS, therefore any variants called for it were assumed to represent heterozygous variants already present in the genome prior to applying the mutagen, and thus most likely shared with the other samples. Tersect 0.12 (Kurowski and Mohareb 2020) was used to remove A1 variants from the other seven variant sets. Variants

appearing in three or more of the samples were also removed, as identical mutations are unlikely to have been induced independently. Finally, the variants were filtered to remove all except SNVs known to be preferentially induced by the EMS mutagen, that is G to A and C to T transitions.

To eliminate likely false positives the two sets of variant calling results were then intersected, retaining only SNVs called by both GATK HaplotypeCaller and bcftools to create "high confidence" sets of SNVs for each mutant. These high confidence sets were then used to estimate the EMS mutation rate related to different concentrations of EMS used for each sample.

3.5.6 Calculating the minimum number of independently derived *Pgt* mutants to confidently identify candidate *Avr.*

For an unannotated genome to be used for comparative mutational genomics, every contig must be treated as if it contains a potential gene candidate. If all the contigs in each genome assembly are of length *l*, have the same GC content, and the canonical EMS mutations are distributed randomly across the assembly with a mutation density *m*, then, following the principles of binomial distribution, the probability (*P*) of a contig in such a genome assembly having mutations in *n* number of mutants by chance alone is:

$P = (lm)^n$

where, *l* = length of the gene (No. of bases)

m = mutation density (No. of SNVs per base of the genome)

n = number of mutants

If the number of contigs of length *l* in such a genome assembly is *G*, then the number of contigs that are likely to have mutations in all *n* mutants by chance alone, *i.e.* the number of false positive candidates, *F*, is:

$F = G \times P$

where, *G* = number of contigs in the assembly

P = probability of a contig in the assembly to have mutations in all the surveyed mutants by chance alone.

Or, $F = G \times (lm)^n$

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Given that the contigs of the UK-01 assembly have a uniform GC-content (**Annexe 9**), and assuming that the EMS SNPs are distributed evenly (Farrell et al. 2014; Shirasawa et al. 2016) the probability of a false positive candidate, is therefore a function of the length of the contig. Analysis of previously sequenced stem rust genomes have estimated that every 10 kb of the genome contains ~2 genes (Duplessis 2011; Li et al. 2019). Thus, contigs of 10 kb or more can potentially complicate the search for candidate genes with mutations in all the mutants, as some of the mutants could have mutations in one gene within the contig while the other mutants could have mutations in the other gene. Therefore, to make our calculations, we used custom python scripts to chop all the contigs that were larger than 5 kb into smaller 5 kb contigs. The resultant chopped assembly contained contigs ranging from 1.001 - 5.999 kb. Since the equation for false positive number calculations only holds for contig lengths of roughly equal sizes, the UK-01 assembly contigs were divided according to their sizes into bins of 100 bp range, i.e. 1000-1100 bp, 1100-1200 bp, 1200-1300 bp and so on. The number of false positives within each bin was calculated using the formula for determining the value of *F,* where *l* = average length of contig in that bin, and *G* = no of contigs in that bin, and *n*=1. In this way, the number of false positives was calculated for each bin and then these values were summed up to give the overall number of false positives from the whole assembly. If the number of false positives equalled a number greater than or equal to 1, then the value of *n* would be increased by 1 and the exercise repeated until the value of *F* was found to be less than 1. The value of *n* for which *F* equals less than 1 is thus considered as the minimum number of mutants required to identify a candidate gene through comparison of re-sequenced genomes of mutants. The GC content of the contigs was calculated using custom code written in Java. The division of the assembly into contig-length bins and iterative calculations of false positives was performed using custom code written in Python.

3.5.7 Screening for gain of virulence on *Sr44*, *Sr43* and *Sr45*.

I conducted screening for gain-of-virulence pustules on each *Sr* line and per mutant batch (**Figure 3.1B** i – ii). For each EMS treatment, sixteen vials of independent mutants were harvested and were pooled into four bulks making a total of 16 bulks of mutants per EMS experiment (**Figure 3.1A** i – iv). Seedlings of introgression lines were grown in a CER and were sown as seven seedlings per pot with eight pots per mutant bulk (128 pots with 856 plants for all bulks) and were treated at day six using the same Maleic hydrazide treatment as described in Chapter 2. For inoculations, 8 mg of dried spores for each *Pgt* bulk and the wildtype used in the inoculations were taken out of cold storage (-80 ^{o}C) and first weighed followed by a heat-shock. The spores were suspended in Novec at a rate of 8 mg of spores in 10 ml Novec per eight pots (56 plants) using an airbrush (**Figure 3.1B** i – ii). The inoculated pots were misted, bagged and then kept under growth conditions described previously. Pustules showing gain-of-virulence phenotypes in comparison to the wildtype control were identified at 16 dpi, dried for four days using silica beads and stored at -80 ^{o}C (**Figure 3.1B** i – ii). They were then used to inoculate the lines on which they were identified upon following a heat shock and resuspension in Novec (**Figure 3.1B** iii – iv). Pustules that maintained their phenotype were then isolated and bulked up on the lines they were identified on for further experiments (**Figure 3.1B** iv– v).

4. Chapter 4. Characterisation of *Puccinia graminis* f. sp. *tritici* gain-of-virulence mutants.

4.1 Abstract.

In this Chapter, I tested whether the *Sr43* and *Sr45 Pgt* gain-of-virulence mutants identified in Chapter 3 would be suitable for sequence analysis in *Avr* gene cloning. To do this, I selected eight and six mutants with a gain-of-virulence on *Sr43* and *Sr45* respectively and inoculated them along with wild type *Pgt* UK-01 onto the stem rust international differential set. Through this, I established that all the mutants maintained the same virulence profile as the wild type suggesting that the acquired virulence was due to the loss of a single *Avr* corresponding to either *Sr43* or *Sr45.* This provided the confidence that the target *Avrs* might be identified via sequence comparison of multiple independently derived virulent mutants with the *Pgt* UK-01 wild type reference.

To determine effect of the loss of *AvrSr43* on fungal fitness, I selected two *AvrSr43* mutants, E1 and E7-1 (M6 and M8 in Fig 3.3, Chapter 3 respectively). These mutants visually exhibited the strongest infection phenotype on *Sr43* which was comparable with that of the wildtype on the susceptible parental line Chinese Spring. The pustule morphology and sporulation also appeared to be unaffected by the mutagenesis (not measured). I quantified the chitin fluorescence in infected *Sr43* leaf tissue alongside the wild type *Pgt* UK-01 at four timepoints: 1 dpi, 2 dpi, 6 dpi and 16 dpi. There were pronounced increases in the chitin fluorescence intensities of E1 and E7-1 growing on *Sr43* compared to those of the wild type inoculated onto *Sr43*. However, there were no differences observed when the mutants on *Sr43* were compared to the wildtype growing on the recurrent parent Chinese Spring. These results showed that loss of *AvrSr43* does not impact pathogen growth as determined by chitin fluorescence. This implies that although *Sr43* is an effective undeployed gene, its resistance might not be durable if cultivars carrying it as a single *R* gene are deployed.

Finally, I conducted microscopy to establish the time of recognition of *AvrSr43* by *Sr43* examining fixed leaf samples inoculated with mutants E1, E7-1 or wildtype. It was not possible to resolve the effect of *AvrSr43* on avirulence from the imaging. Analysis of counts of invasive fungal structures from microscopy images showed no significant difference in the growth and development of the virulent mutants and wildtype *Pgt* at 1 dpi and 2 dpi. However, detection

of reactive oxygen species (ROS) production by 3,3'-Diaminobenzidine (DAB) staining combined with additional sampling intervals resolved the time point of *AvrSr43*/*Sr43* interaction to be 3 days post inoculation.

4.2 Introduction.

To enable successful parasitism, plant pathogens secrete effectors, a wide range of molecules that modify the host cell environment and suppress innate immunity (Oliva et al. 2010). A subset of these fungal effectors have a gene-for-gene relationship with resistance (*R*) genes in their plant hosts that directly recognise the presence of specific effectors or their interaction with plant cell targets and thereby induce defence responses (Dodds and Rathjen 2010). These effectors are termed avirulence effectors (Avr) and the collective responses induced due to recognition by their corresponding *R* genes is called effector-triggered immunity (ETI). A hallmark feature of ETI is the rapid production of reactive oxygen species which induces rapid localized cell death termed the hypersensitive response (HR) (Dodds and Rathjen 2010).

However, some *Avrs* can serve to prevent the recognition of *other Avrs* hence disrupting the model of ETI in the presence of a corresponding *R* gene. For example, the *L. maculans AvrLm3* is recognized by the *R* gene *Rlm3*. This recognition is suppressed when *AvrLm4-7* is present whilst *AvrLm4-7* itself can be recognized by two resistance genes, *Rlm4* and *Rlm7*. Thus, silencing of *AvrLm4-7* in an isolate virulent toward *Rlm3* results in avirulence against *Rlm3*, and transformation of *AvrLm4-7* into an isolate avirulent on *Rlm3* leads to virulence toward cultivars with *Rlm3* (Plissonneau et al. 2016). These interactions can affect identification of an Avr of interest via mutational genomics as this would require the knocking out of two genes in multiple candidates and this would have a very low probability of occurring.

Pathogens can become virulent against hosts carrying *R* genes because of evolution of their corresponding *Avr* gene complement. This can occur as a result of deletion, disabling, or suppression of expression of the *Avr* gene (Jonathan D G Jones and Dangl 2006). In addition,

single nucleotide polymorphisms (SNPs) can result in the *Avr* no longer being recognised by its corresponding *R* gene whilst still retaining its virulence function (Guttman et al. 2014).

The AvrLm1 in *Leptosphaeria maculans, which causes blackleg on Brassica crops,* is recognised by *Rlm1*. Isolates virulent against cultivars carrying *Rlm1* carry a deletion of *AvrLm1. These isolates were discovered* within a short period after deployment of cultivars with *Rlm1* suggesting that loss of *AvrLm1 does not have a significant impact on fungal fitness* (Gout et al. 2007). However, the durability of resistance conferred by *Rlm7* gives an indication of the importance of *AvrLm4-7* for *L. maculans* fitness as it retains the effector despite the selection pressure. The *L. maculans AvrLm4-7* is recognized by both *Rlm4* and *Rlm7 (Delourme et al. 2004)*. *Rlm4* was widely deployed from the 1970s and virulence towards it is now widespread (Rouxel and Balesdent 2017). This virulence was attributed to a non-synonymous SNP mutation without a change in the overall 3-D structure of AvrLm4-7 (Blondeau et al. 2015). However, this has not affected its recognition by *Rlm7* (Parlange et al. 2009a). Since 2004, *Rlm7* cultivars make up between 50–70% of the oilseed crop in France (Balesdent et al. 2015). Despite this, evolution of virulence amongst isolates increased marginally over a long period from 4% in 2014 to 19% in 2013. This involved a combination of deletions and three amino acid substitutions with minor changes in the 3-D structure (Daverdin et al. 2012; Blondeau et al. 2015).

In rusts, deletion of *Avrs* can result in new virulence towards *R* genes. The Australian isolate *Pgt*632 is a spontaneous mutant which is virulent to *Sr50*. This isolate resulted from the loss of a 2.5 Mb chromosome segment by *Sr50*-avirulent *Pgt*279 (Chen 2017). Manual inspection of the lost region showed 18 haustorially secreted protein genes with non-synonymous variation between the *Pgt*279 and *Pgt*632 isolates (Chen 2017). This resulted in *Pg*t279 carrying two gene alleles, while *Pgt*632 contained only one allele. The spontaneous gain-of-virulence was a result of a loss-of-heterozygosity for the effector candidates. Through sequence comparison and effector prediction, *AvrSr50* was identified and more recently, *AvrSr27* (Chen et al. 2017; Figueroa et al. 2020). At the time of writing, there was no information on the prevalence of *Pgt*632 in Australia or regarding its fitness after losing multiple *Avrs*.

For *Magnaporthe oryzae,* the causal agent of rice blast, isolates virulent against rice plants carrying the resistance gene *Pia* were established to have a SNP in *AVR-Pia, which* leads to loss of recognition (Cesari et al. 2013a). In the pathogen *Fusarium oxysporum f*. sp. *lycopersici, which causes vascular wilt on tomato, Avr1* is involved in the suppression of the resistance conferred by the *I-2/Avr2* or *I-3*/*Avr3* interaction. *Avr1* deletion mutants are recognised by plants carrying *I-3* or *I-2* whilst addition of *Avr1* to isolates controlled by these *R* genes resulted in virulence (Houterman et al. 2008). In the absence of the corresponding *R* genes, *Avr2* and *Avr3* have been determined to be critical for virulence whilst loss of *Avr1* did not impact on virulence (Houterman et al. 2008). Thus, in some cases, loss of *Avr* function has no negative impact on fungal pathogen virulence and fitness whereas in other cases there is a negative effect. The ability of a fungal pathogen to successfully infect its host and complete its lifecycle after the loss of an Avr can be attributed to the functional redundancy of the multitude of effectors which are secreted simultaneously during infection.

For a few *Avrs*, deletion or mutation has been shown to negatively impact virulence. In the absence of I2, the movement of *Fol* Avr2 from cell to cell to promote virulence is facilitated by the effector Six5 that changes plasmodesmata exclusivity (Cao et al. 2018). In *C. fulvum*, the effector Avr4 contains a chitin-binding domain that binds to and protects the cell walls of chitin-containing fungal pathogens from hydrolysis by plant chitinases (Van Den Burg et al. 2006). This effector is also present in *F. solani* and *Trichoderma* and its chitin-binding activity prevents release of chitin molecules that trigger immunity. In *Verticillium albo-atrum* the effector gene *Ave1* which corresponds to the resistance gene *Ve1* in tomato, and which is required for full aggressiveness of Verticillium on tomato plants lacking *Ve1* (De Jonge et al. 2012).

The loss of *Avr* genes is not always associated with any detectable phenotypes apart from gain-of-virulence. In the bacterium *Pseudomonas syringae* pv. *tomato* DC3000, deletion of effectors to determine their significance in virulence was investigated (Cunnac et al. 2011). Loss of single effectors did not have an impact as single effectors had multiple targets associated with defence within the host (Cunnac et al. 2011). This could have been a result of functional redundancy for the virulence function of effectors, whereby knocking out one had no discernable effect on phenotype on a susceptible host. However, a core set of eight

effectors, AvrPtoB, HopM1, HopE1, HopG1, HopAM1-1, AvrE, HopAA1-1, and HopN1, was established to be adequate for growth and virulence (Cunnac et al. 2011). Concerning fungi and oomycetes, however, the impact of deletion of *Avrs* has been difficult to determine. This is due to effector functional redundancy observed in several pathosystems. This has implications on the durability of resistance genes corresponding to such avirulence effectors.

In this Chapter, I conducted differential analysis on selected mutants to determine whether the eight and six gain-of-virulence mutants obtained in Chapter 3 had lost only their avirulence against *Sr43* or *Sr45*, respectively. Following this, I examined the effect of *AvrSr43* loss on the viability of *Pgt* during infection of wheat in the presence and absence of *Sr43*. To do this, I used the wheat germ agglutinin chitin (WAC) assay which uses chitin fluorescence as a proxy to determine fungal biomass *in planta*. (Ayliffe et al. 2013). I also took samples from these inoculations to investigate the effect of *Sr43* on wildtype *Pgt* growth using confocal microscopy to view fungal growth at four time points. Finally, I used 3,3'-Diaminobenzidine (DAB) to stain for peroxide production produced during the HR response to establish when AvrSr43 is detected during infection of wheat carrying *Sr43*.

4.3 Results.

4.3.1 *Pgt* mutants virulent on *Sr43* and *Sr45* maintain the same virulence profile as the wild type on the international standard differential set.

I tested whether the gain-of-virulence mutants obtained in Chapter 3 had lost only their avirulence against *Sr43* or *Sr45*. To do this, I randomly selected eight and six mutants with a gain-of-virulence on *Sr43* and *Sr45*, respectively, and inoculated them as well as wild type *Pgt* UK-01 onto the stem rust international differential set. This set was originally developed by Stakman (1962) and had 16 *Sr* lines. This was updated to twenty *Sr* lines (Jin et al. 2008b). I conducted phenotyping to determine the infection from 14 to 16 dpi (**Table 4.1**). Wild type *Pgt* UK-01 and the gain-of-virulence mutants identified on *Sr43* and *Sr45* were avirulent on three (*Sr11*, *Sr24* and *Sr31*) out of the 20 lines in the set. The infection types of the 14 mutants were consistent with that of *Pgt* UK-01 wild type. Thus, the mutants had the same race designation of TKTTF as defined by their virulence profile on the differential set. The mutants

showed no additional virulence to the other 17 *Sr* lines in the set and only showed added virulence on either *Sr43* or *Sr45.*

Table 4.1 | ***Pgt* race profiles of randomly selected mutants virulent on *Sr43* or *Sr45**.**

				***Sr43*[e] M-1**	***Sr43* M-3**	***Sr43* M-4**	***Sr43* M-5**	***Sr43* M-6**	***Sr43* M-7**	***Sr43* M-8**	***Sr43* M-9**	**Race[h]**
			UK-01[d]	**Sr45[f] M-1**	***Sr45* M-2**	***Sr45* M-3**	***Sr45* M-4**	***Sr45* M-5**	***Sr45* M-6**			
Set[a]	**Differential line ID[b]**	**Gene[c]**	Infection type High (> 2+) or Low (<2+)[g]									
1	ISr5-Ra CI 14159	*Sr5*	H	H	H	H	H	H	H	H	H	**T**
	*T monococcum/8*LMPG-6 DK13*	*Sr21*	H	H	H	H	H	H	H	H	H	
	Vernstein PI 442914	*Sr9e*	H	H	H	H	H	H	H	H	H	
	ISr7b-Ra CI 14165	*Sr7b*	H	H	H	H	H	H	H	H	H	
2	Yalta PI 155433	*Sr11*	L	L	L	L	L	L	L	L	L	**K**
	ISr6-Ra CI 14163	*Sr6*	H	H	H	H	H	H	H	H	H	
	Mentana W1124 PI 221154	*Sr8a*	H	H	H	H	H	H	H	H	H	
	Acme CI 5284	*Sr9g*	H	H	H	H	H	H	H	H	H	
3	W2691SrTt-1 CI 17385	*Sr36*	H	H	H	H	H	H	H	H	H	**T**
	Prelude*4/2/Marquis*6/Kenya 117A	*Sr9b*	H	H	H	H	H	H	H	H	H	
	Festiguay W2706 PI 330957	*Sr30*	H	H	H	H	H	H	H	H	H	
	Prelude/8*Marquis*2/2/Esp 518/9	*Sr17*	H	H	H	H	H	H	H	H	H	
4	ISr9a-Ra CI 14169	*Sr9a*	H	H	H	H	H	H	H	H	H	**T**
	ISr9d-Ra CI 14177	*Sr9d*	H	H	H	H	H	H	H	H	H	
	W2691Sr10 CI 17388	*Sr10*	H	H	H	H	H	H	H	H	H	
	CnsSrTmp	*SrTmp*	H	H	H	H	H	H	H	H	H	
5	LcSr24Ag	*Sr24*	L	L	L	L	L	L	L	L	L	**F**
	Kavkaz/Federation4	*Sr31*	L	L	L	L	L	L	L	L	L	
	VPM1	*Sr38*	H	H	H	H	H	H	H	H	H	
	McNair 701 (CI 15288)	*SrMcN*	H	H	H	H	H	H	H	H	H	

*Please refer to the table legend below explaining the column headings

Table legend:

a, b and c: The International Stem Rust Differential Set is composed of five sets (a) containing four specific cultivars (b) each carrying a single, defined *Sr* gene (c) (Jin et al. 2008).
d, e and f: Five plants of each differential line were inoculated with the wildtype UK-01 (d), eight EMS derived mutants of UK-01 virulent on *Sr43* (e) and six virulent on S*r43*. The experiment was conducted twice.
g: The infection types were determined as laid out by Stakman et al. (1962) through visual scoring of pustule size. The infection types were then graded as high (H) if greater than size 2+ or low (L) if below the size of 2+.
h: A matrix (Annexe 12) is used to determine the letter denoting the race of the isolate based on the infection types on the lines in each set (Stakman et al. 1962).

4.3.2 Loss of *AvrSr43* does not affect *Pgt* UK-01 fitness.

To determine the effect of *Avr* loss on the viability of *Pgt* during infection of wheat I used the wheat germ agglutinin chitin (WAC) assay to quantify the fungal biomass *in planta* via fluorescence (Ayliffe et al. 2013). The WAC assay quantifies fungal biomass *in planta* by detecting fluorescence. Fungal chitin is stained using wheat germ agglutinin conjugated to a fluorophore like alexa488 (WGA AF488). Plant tissue is collected at selected time points of the inoculation experiment. Weighed infected plant tissue is cleared, macerated, stained, then fluorescence is measured. Fluorescence intensity exhibits a linear relationship with the amount of chitin until the quantity of the stain added is limiting (Ayliffe et al. 2013). Chitin is a component of the cell walls of several fungi. Thus, this assay enables simple and accurate measurement of biomass accumulation in some plant-fungal interactions.

I selected two *AvrSr43* mutants, E1 and E7-1 (M-6 and M-8 in Fig 3.3, Chapter 3 respectively). These mutants visually exhibited the strongest infection phenotype on *Sr43* which was comparable with that of the wildtype on the susceptible parental line Chinese Spring. The pustule morphology and sporulation also appeared to be unaffected by the mutagenesis (not measured. In addition, the wildtype strain was also inoculated onto Chinese Spring, the susceptible recurrent parent of the *Sr43* introgression line. Tissue samples were collected at four time points, cleared of chlorophyll, ground, stained with WGA-AF488 and fluorescence was recorded from aliquots of the macerated tissue using a plate reader. I quantified chitin fluorescence in infected *Sr43* leaf tissue alongside the wild type *Pgt* UK-01 at four timepoints: 1 dpi, 2 dpi, 6 dpi and 16 dpi. There were pronounced increases in the chitin fluorescence intensities of E1 and E7-1 growing on *Sr43* compared to those of the wild type inoculated onto

Sr43. However, there were no differences observed when the mutants on *Sr43* were compared to the wildtype growing on the recurrent parent Chinese Spring. These results showed that loss of *AvrSr43* does not impact pathogen growth as determined by chitin fluorescence. This implies that although *Sr43* is an effective undeployed gene, its resistance might not be durable if cultivars carrying it as a single *R* gene are deployed.

Fungal chitin fluorescence for the gain-of-virulence mutants E1 (**P < 0.01) and E7-1 (*P < 0.05) were significantly higher than that of the wild type on *Sr43* in both experiments (Annexes 15 and 16). In the absence of *Sr43*, fungal growth of the wild type *Pgt* UK-01 as shown by the fluorescence readings on the recurrent parent Chinese Spring was marginally higher, but not significantly different to that of the mutants on *Sr43* (Figures 4.1 A and 4.1 B; Annexes 15 and 16). There was no apparent difference in chitin fluorescence between E1 on *Sr43* and wild type *Pgt* on the susceptible line at 1 and 2 dpi (Fig 4.1 A, Annexe 15). The growth rate of E1 on *Sr43* from 2 dpi to 6 dpi was 2.2-fold whilst it was 1.7-fold for E7-1 on *Sr43.* Between 6 dpi and 16 dpi, the rate of increase in chitin content was 1.2-fold and 1.4-fold respectively. In addition, the trend in fluorescence over time for the wildtype on *Sr43* increased marginally between 2 dpi and 6 dpi for both experiments. The gain-of-virulence mutant E7-1 showed a similar pattern to mutant E1 on *Sr43* where the amount of chitin fluorescence was higher than that of the wild type on the resistant line being 1.5-fold at 6 dpi and 1.7-fold at 16 dpi (Fig 4.1 B, Annexe 16).

Overall, increase in chitin fluorescence readings of tissue from the line carrying *Sr43* inoculated with mutant E1 increased 6-fold whereas chitin fluorescence for the wild type inoculated on the same line increased 1.8-fold from 1 dpi to 16 dpi. In the case of E7-1, fluorescence intensity increased 3.3-fold from 1 dpi to 16 as opposed to 1.7-fold for the wild type on the same line. In the absence of *Sr43* (i.e. on Chinese Spring), a similar increase in readings was observed for the wild type as that observed for the mutants on the *Sr43* introgression line.

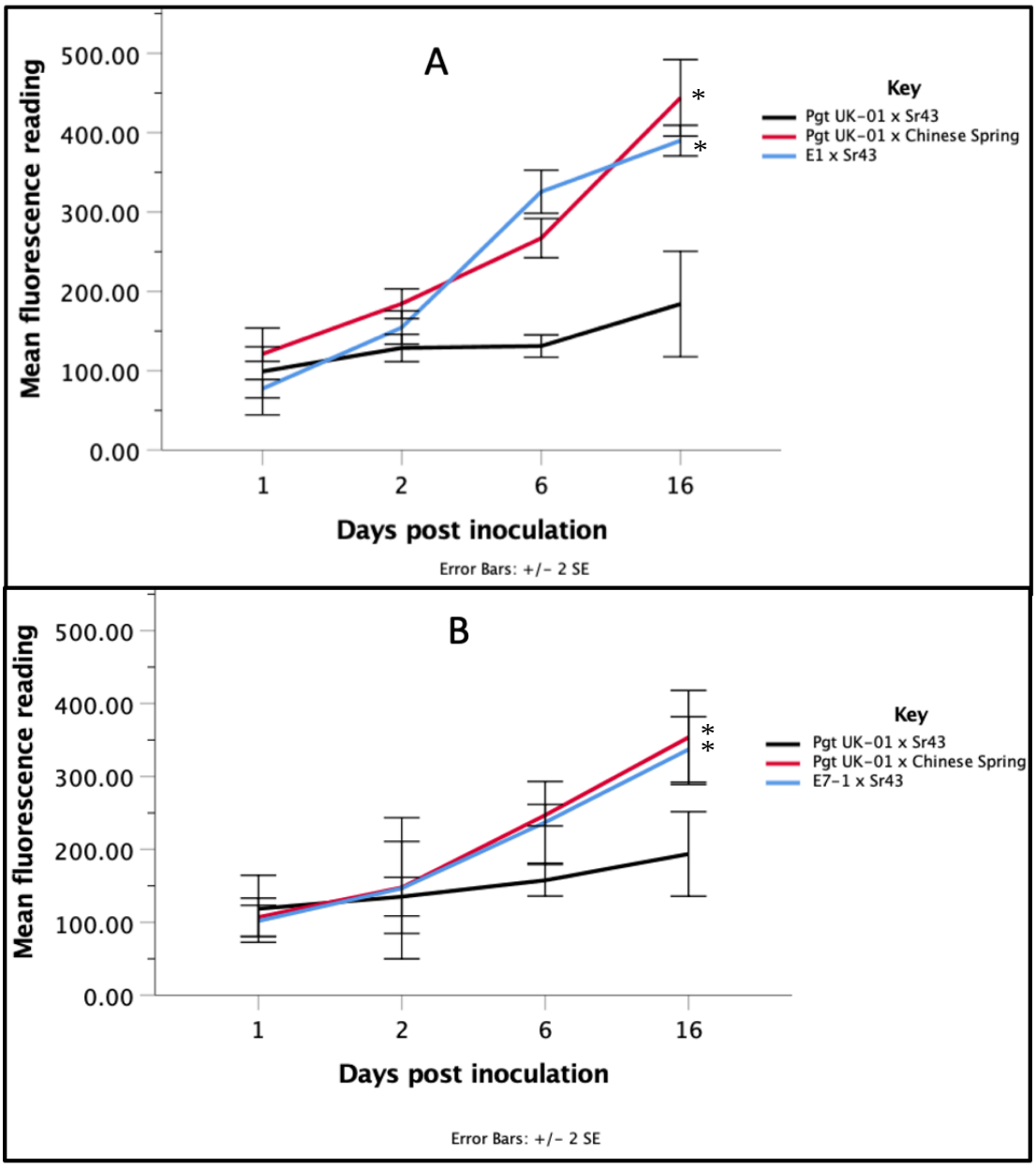


Figure 4.1 | ***In planta*** **chitin fluorescence readings of *Pgt* UK-01 and mutants E1 and E7-1 at four time points.**

Chitin fluorescence for mutant E1 (panel A) and mutant E7-1 (panel B) on the resistant Sr43 line compared to the wild type on Sr43 and the recurrent parent Chinese Spring. Asterisks denote significantly different, (**P < 0.01 (panel A) and *P < 0.05 (panel B), from the fluorescence readings of wild type *Pgt* Uk-01 on *Sr43*).

4.3.3 There are differences in wildtype and mutant *Pgt* growth patterns are at 1 and 2 days post inoculation.

To determine the effect of *Sr43* on the growth and development of *Pgt* UK-01, I inoculated the *Pgt* UK-01 wildtype onto the wheat-*Thinopyrum ponticum* introgression line *Sr43* and its recurrent parent Chinese Spring (CS). For comparison, I inoculated the two gain-of-virulence mutants E1 and E7-1, that were found not to exhibit any apparent deleterious effects from their background EMS mutations on their growth and development (Figure 4.1). I collected and fixed samples from all treatments for confocal imaging at 1, 2, 6 and 16 dpi (Fig 4.2). Images of samples were recorded at depth intervals of 1 µm throughout the entire sample volume to allow for complete visualisation of fungal infection structures within the leaf tissue. Propidium iodide could not determine the stage of cell death as the tissue was fixed before staining. However, it was useful in visualising plant cell walls and stomata.

Between eight and 25 optical sections (Z-stacks) were recorded per treatment and then combined to produce a composite image. At 1 and 2 dpi there was no evidence of *Sr43* genetically interacting with *AvrSr43* to suppress spore germination, growth of external hyphae, or the formation of appressoria, sub-stomatal vesicles, intercellular hyphae, and haustoria (Fig 4.2). At 6 dpi, the number of haustoria of the wildtype on *Sr43* increased but there was no pustule development evident (Fig. 4.2 I). In contrast, *Pgt* wildtype growth on Chinese Spring (panel L) as well as mutants E1 (panel J) and E7-1 (panel K) at the same timepoint showed pustule establishment. The growth of spores on the uredia pushed against the leaf epidermis for E1 on *Sr43* (panel J) and wildtype on Chinese Spring (panel L) whilst the uredia for mutant E7-1 had ruptured the epidermis (panel K). At 16 dpi, all treatments had developed mature pustules, but pustule size of the wildtype on *Sr43* was smaller compared with its growth on Chinese Spring and the gain-of-virulence mutants on *Sr43*.

I quantified the number of invasive structures from six to ten representative confocal images at 1 dpi and 2 dpi across all the inoculations to determine if *Sr43* had any negative effect on the infection of the wildtype. I counted structures present at various stages of infection from spore gemination, number of hyphae on the leaf surface, formation of appressoria and sub-stomal vesicles, intercellular hyphae and haustoria formation. I plotted the counts for number of hyphae on the leaf surface, number of intercellular hyphae and number of haustoria (Fig

4.3). Analysis of variance indicated that the number of structures per treatment were not significantly different ($P > 0.05$). Means comparison by Fishers LSD also showed no effect of treatment at 1 dpi and 2 dpi. At 6 dpi, counting could only be conducted for the wildtype on *Sr43* which had not yet established pustules whereas the wildtype on Chinese Spring and the *Pgt* mutants on *Sr43* had formed extensive networks of hyphae and uredia.

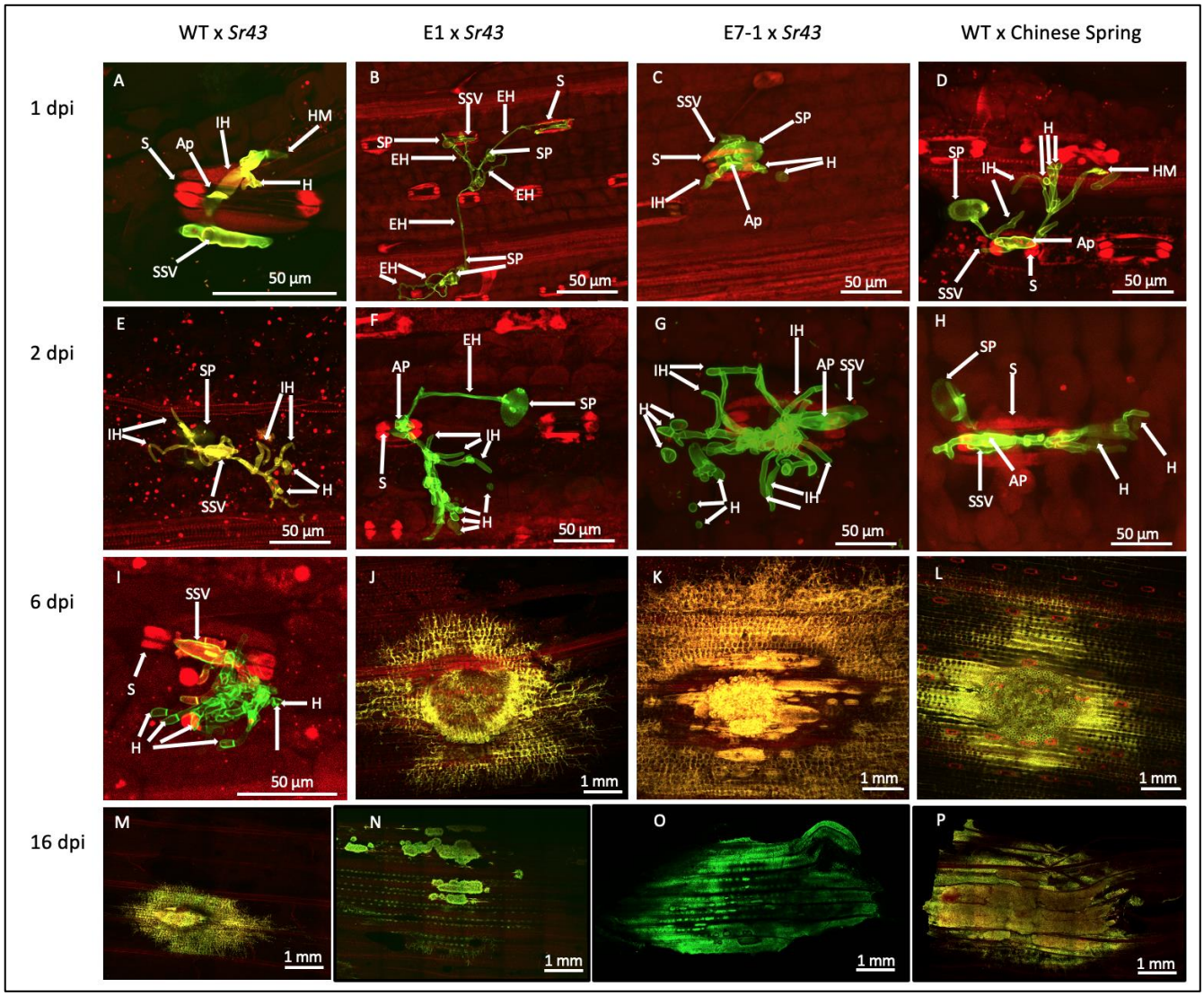


Figure 4.2 | Development of invasive structures of *Pgt* UK-01 wildtype (WT) inoculated onto the introgression line *Sr43* (panels A, E, I, M) and its recurrent parent Chinese Spring (panels D, H, L, P). *Pgt* UK-01 gain-of-virulence mutants E1 (panels B, F, J, N) and E7-1 (panels C, G, K, O) were inoculated onto *Sr43*.

Samples were collected for imaging at 1, 2, 6 and 16-days post inoculation. Leaf tissue was first cleared with ethanol:trichloromethane (3:1, v/v) containing 0.15% (w/v) trichloroacetic acid to remove chlorophyll before co-staining with WGA AF4888 and Propidium Iodide. Around 10 images were viewed per treatment. No differences were observed between the wildtype and the mutants at 1 and 2 dpi in terms of germinated spores (SP), external hyphae (EH), appressoria (AP), stomata (S) entry, sub-stomatal vesicles (SSV), intercellular hyphae (IH), haustoria mother cells (HM) and haustoria (H). At 6 dpi, colony establishment and spore development below the epidermis was evident except for the wildtype on *Sr43*. Pustules emerging from the leaf epidermis were visible for all treatments at 16 dpi with the wildtype on the resistant line forming smaller infection sites compared to the wildtype on Chinese Spring and mutants E1 and E7-1 on the resistant line (*Sr43*).

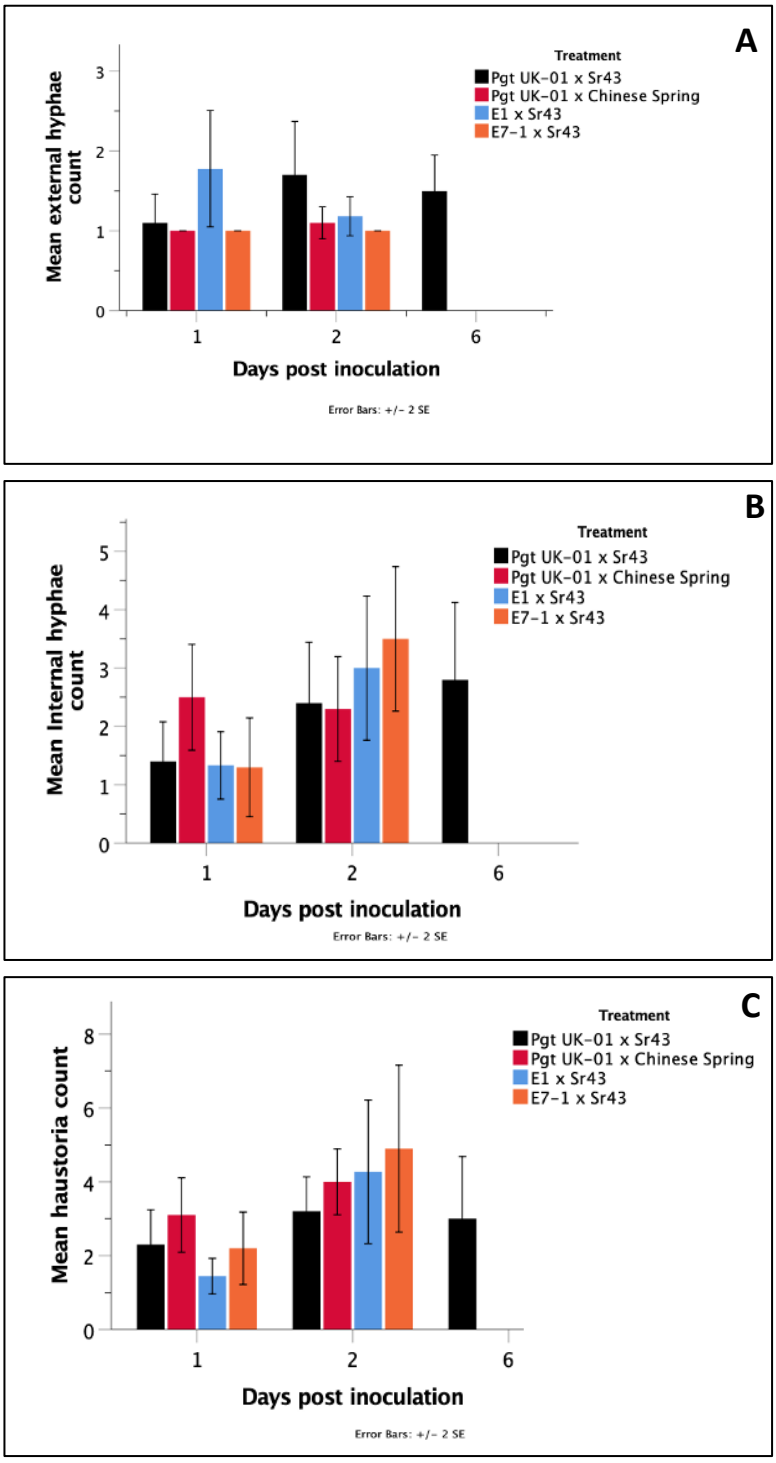


Figure 4.3 | Number of germinated spores (A), intercellular hyphae (B) and haustoria (C) counted from 10 confocal images each of wildtype *Pgt* UK-01 on Sr43 and Chinese Spring as well as Pgt mutants E1 and E7-1 on Sr43 at one, two- and six-days post-inoculation.

No significant differences ($P > 0.05$) in the counts of infection structures could be established for any of the treatments at 1 dpi and 2 dpi. At 6 dpi, infection structures could only be quantified for wildtype Pgt on Sr43 while in the other samples, the infection structures were masked by colony establishment and sporulation (Fig 4.2).

4.3.4 Staining for reactive oxygen species indicates that AvrSr43 is detected by 3 days post inoculation.

To resolve the time point where *AvrSr43* is detected by *Sr43*, I tested for ROS production as an indicator of the *Sr*/*Avr* interaction. I conducted the four previously mentioned inoculation treatments as in the microscopy experiments (*Pgt* UK-01 x *Sr43*, *Pgt* UK-01 x Chinese Spring, E1 x *Sr43* and E7-1 x *Sr43*). I collected samples at timepoints 1, 2, 3, 5, 6 and 16 dpi. Detection of ROS was determined by the brown precipitate of 3,3'-Diaminobenzidine (DAB) around the infection lesions on the leaves (Fig 4.4). These were visible from 3 dpi, to 16 dpi on the leaf samples of the wildtype treatment on *Sr43* whilst none were visible for the wildtype on Chinese Spring, E1 on Sr43 and E7-1 on *Sr43* between 1 and 6 dpi. At 16 dpi the DAB precipitate was observed across all treatments and its pattern of deposition followed the diamond shape that is characteristic of *Pgt* pustules (Fig 4.4).

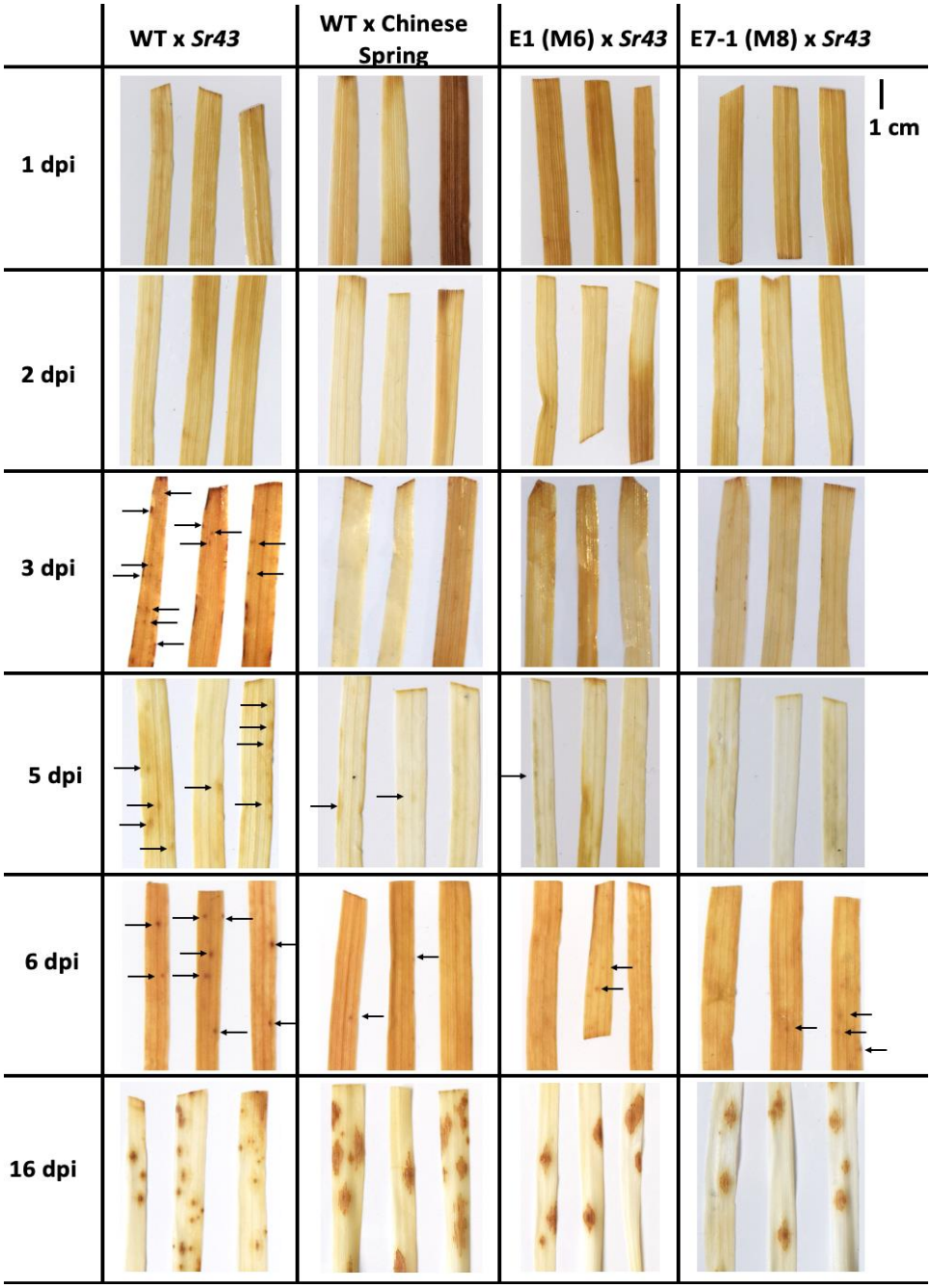


Figure 4.4 | Leaf segments stained with 3,3'-Diaminobenzidine (DAB) to detect ROS production in response to infection by the wildtype *Pgt* UK-01, and virulent mutants E1 and E7-1 on *Sr43*, plus wildtype *Pgt* UK-01 on Chinese Spring (-*Sr43*).

For each treatment, 56 plants were inoculated as following: wildtype (WT) x *Sr43*, WT x Chinese Spring (-*Sr43*), E1 x *Sr43* and E7-1 x *Sr43*. From these plants, three samples were taken per treatment for ROS staining at timepoint 1, 2, 3, 5, 6, and 16 dpi. The earliest detection of ROS was at 3 dpi through to 16 dpi for wildtype *Pgt* UK-01 on *Sr43* whilst ROS were detected mainly at 16 dpi for the remaining treatments where *Sr43* was inoculated with virulent mutants and on Chinese Spring (-*Sr43*) inoculated with the wildtype. Arrowheads indicate sites of ROS staining (brown spots) in earlier time periods. At 16 dpi, all pustules were stained

with DAB with smaller areas on *Sr43* x WT avirulence interaction. This experiment was conducted twice.

4.4 Discussion.

The cloning of genes via mutational genomics is dependent on the identification of multiple independently derived individuals with loss of function in the target gene (Steuernagel et al. 2017). In the case of the EMS mutants derived from *Pgt* UK-01, I identified multiple individuals with gain-of-virulence to either *Sr43* (nine mutants) or *Sr45* (14 mutants). In this chapter, I conducted race typing of eight and six mutants with virulence towards *Sr43* and *Sr45*, respectively, on the stem rust international differential set containing lines carrying 20 single *Sr* genes. From this, I established that the mutants retained the same TKTTF race designation as the wild type parent (Table 4.1). These results confirmed the virulence towards either *Sr43* or *Sr45* observed was likely due to loss of *AvrSr43* or *AvrSr45* alone. The loss of multiple *Avrs* would complicate bioinformatics analysis to identify causal mutations. Moreover, the race analysis confirmed that the virulence observed was not the result of contamination by natural isolates virulent to *Sr43* or *Sr45*. *Pgt* UK-01 was the only isolate used in the laboratory where my experiments were conducted and *Pgt* UK-01 is the only known isolate present in the UK (Lewis et al. 2018).

In this study, the general conservation in virulence profiles between the *Pgt* wild type isolate and mutants also provides further support to these being genuine derived mutants rather than potential contaminants. In the cloning of *AvrSr35* by mutational genomics similar results were obtained where EMS mutants derived from the *Pgt* race RKQQC maintained the same virulence profile as the wildtype (Salcedo et al. 2017). In contrast, Li *et al.* (2019) reported that EMS mutagenesis of the *Puccinia striiformis* f. sp. *tritici* (*Pst*) race PSTv-18 resulted in 33 individual mutants with virulence towards multiple genes. Wildtype PSTv-18 is avirulent to all the 18 lines in the *Pst* differential set. Race analysis of these mutants determined 24 different *Pst* races. Such drastic changes in virulence pattern for so many mutants (24 out of 33) suggest that these are more likely environmental contaminants rather than bona fide EMS-derived mutants. The observation that the 33 *Pst* mutants reported in the Li et al. study contained a much higher number of average SNVs (24,796) compared to the number of mutations

detected in my 23 *Pgt* mutants (659 for high confidence SNVs and 3,287 for non-redundant SNVs) supports this notion.

Loss of *AvrSr43* did not have any noticeable impact on pathogen fitness as shown by the chitin fluorescence readings from leaf tissue of the wheat introgression line carrying *Sr43* infected by mutants E1 and E7-1. These mutants are derived from the highest EMS concentration of 0.075 M. Therefore, the high background mutation densities at this EMS concentration (3,855 SNVs) (*see* Chapter 3) did not affect fungal growth and development in these two mutants. Wheat germ agglutinin AlexaFluor 488^{TM} conjugate has a high binding specificity for chitin thus enabling fluorescence intensity of stained leaf tissue to be an indication of the extent of fungal growth in the leaf tissue (Ayliffe et al. 2013). Chitin fluorescence readings of the wildtype on *Sr43* was significantly lower than on Chinese Spring (which does not contain *Sr43*) and those of the mutants on the Chinese Spring-*Sr43* introgression line.

Time permitting, I could have conducted additional experiments to conclusively determine that the loss of *AvrSr43* does not have any identifiable fitness cost on *Pgt* UK-01. Methods like quantification of fungal biomass via a biochemical proxy such as ergosterol can measure living cells only. This is opposed to evaluating the cell wall content I used in this study (Newell 2001). qPCR can also quantify fungal biomass as done for *Aspergillus flavus* using primers for Membrane protein, Elongation Factor 1 alpha and Beta tubulinM genes (Mitema et al. 2019). In some cases, RT-PCR can be used to detect and quantify two fungi as for the co-infections of *Pyrenophora tritici repentis* and *Parastagonospora nodorum* (Abdullah et al. 2018). This would also enable experiments exploring the competitiveness of the gain-of-virulence mutant isolates when inoculated on the same plants along with the wildtype. However, RT-PCR methods require optimisation, and the WAC assay was simple to use and a direct approach in quantifying chitin, a major component of many fungal cells. The WAC assay thus gave preliminary evidence as the role of *AvrSr43* in Pgt UK-01 fitness.

In my experiments, I did not compare the wildtype and virulent isolates on the same genetic background. The wildtype was grown on Chinese Spring which is the recurrent parent of the line carrying *Sr43.* Mutant *sr43* lines were present. I, however, could not use them due to a conflict of interest with another project. Alternatively, I could have compared the mutant

isolates with the wildtype on Chinese Spring. However, I used *Sr43* since the isolates were virulent towards it. In addition, inoculating the wildtype on *Sr43* allowed me to establish the effect of *Sr43* on fungal growth. From this, I could also determine when *AvrSr43* was detected using the DAB assay.

This difference in fungal growth can thus be attributed to the effect of *Sr43* genetically interacting with *AvrSr43*. The ability of the mutants to grow on *Sr43* as well as the wildtype on Chinese Spring shows that loss of the avirulence effector is well tolerated. This can be attributed to the functional redundancy of effectors in fungi and oomycetes. Deletion of 78 effectors in *Magnaporthe oryzae* and six related effector genes in *Ustilago maydis* did not have any major effect on pathogen growth (Farfsing et al. 2005; Saitoh et al. 2012). In contrast, deletion of *Pep1* in *U. maydis* stopped development due to uninhibited defences (Hemetsberger et al. 2012b), while the effector Avr2 in *Cladosporium fulvum* was found to be necessary for full virulence against susceptible tomato species (van Esse et al. 2008), and loss of the *Blumeria graminis* f. sp. *tritici* candidate effector CSEP005 inhibited penetration (Zhang et al. 2012).

In my microscopy experiments, it was not possible to use confocal microscopy to determine the timepoint when cell death occurred due to detection *AvrSr43* by *Sr43*. Propidium iodide (PI) stain, is appropriate for detecting dead cells because it cannot cross the membranes of living cells. However, due to the process of fixing leaf samples for microscopy using a solution of chloroform, ethanol and trichloroacetate, PI could infiltrate the cells thus staining the nuclei. Live cell imaging, which is done without fixing of the tissue would have been the more appropriate method to determine the point of cell death initiated by recognition of AvrSr43 as PI would only have penetrated the cells where HR would have occurred and the apoplast.

Detection of reactive oxygen species (ROS) by 3,3' DAB staining was therefore used to determine the time of *Avr*/*Sr* interaction. Under stress conditions such as pathogen invasion rapid production of ROS known as an oxidative burst can occur as a result of pathogen recognition by the products of *R* genes (Thordal-Christensen et al. 1997). This accumulation of ROS disrupts plant cell membranes, resulting in programmed cell death (Wojtaszek 1997). DAB is polymerised by reacting with the superoxide radicals, hydrogen peroxide and hydroxyl

radical, to form an insoluble brown precipitate where pathogen penetrating structures such as appressoria and haustoria invade host cells; these are the sites of effector secretion by the fungus into the plant cytoplasm (Liu and Friesen 2012). The DAB precipitate was visible at 3, 5, 6 and 16 dpi on the leaf samples of *Sr43* inoculated with wildtype *Pgt* UK-01 (Fig 4.3). This suggests the time of expression and recognition of *AvrSr43* starts at 3 dpi and could explain why there were no noticeable differences in wildtype and virulent mutant *Pgt* on *Sr43* at 1 and 2 dpi. At 16 dpi ROS production amongst all treatments could be attributed to plant stress due to pathogen growth and expansion as uredinia rupture mesophyll and epithelial tissue. In other pathosystems detection of ROS production by DAB staining in non-host resistance to *Blumeria graminis* f. sp. tritici (*Bgt*) in Barley occurs around appressoria at 18 hours after inoculation (Huckelhoven et al. 2001). In wheat inoculated with *P. striiformis* f. sp. *tritici*, ROS production was detected between 12 and 24 hours post inoculation in guard cells during appressorium formation and in mesophyll cells after development of haustoria. In my experiments, *Pgt* UK-01 formed appressoria and haustoria starting at 1 dpi (Fig 4.2 A – D) but ROS was not detected until 3 dpi (Fig 4.4).

Sr43 is a non-canonical *R* gene, i.e. it does not encode a nucleotide-binding and leucine-rich repeat (NLR) protein (Yu et al. 2020). In this respect, it was interesting to establish that it elicits a hypersensitive resistance response typical of an NLR. The detection of *AvrSr43* at 3 dpi does not completely halt development of the fungus. Rather, it restricts the final pustule size as demonstrated in the inoculations of *Pgt* UK-01 on *Sr43* Chapters 2 (Fig 2.3) and the microscopy images (Fig 4.2 I and M)

In summary, recognition of *AvrSr43* by *Sr43* is associated with a hypersensitive response as shown by ROS detection at 3 dpi. Loss of *AvrSr43* does not have a significant impact on the fitness of *Pgt* UK-01. Therefore, stacking *Sr43* with *Sr* genes that recognise other effectors is necessary since the resistance conferred by *Sr43* deployed as a single gene in a cultivar can be easily overcome.

4.5 Methods

4.5.1 Determining *avrSr43* and *avrSr45* mutant race profiles on the stem rust international differential set.

Virulent pustules identified on *Sr43* and *Sr45* were purified via single pustule isolation and multiplied as described in Chapter 3 in preparation for inoculation of the stem rust differential set. I prepared five 12 to 14-day-old seedlings of each of the 20 standard *Sr* lines within the differential set. Inoculum was applied to the leaves of the plants at a rate of 8 mg (total 20 mg for 20 pots) of the wildtype urediniospores per eight pots. The spores were first "heat shocked" in a water bath at 45 ^{o}C for 10 minutes. I suspended the 8 mg of spores in 10 ml of 3M® NOVEC 7100® and immediately inoculated the respective cultivars (Table 4.1) in a Class 2 biological safety cabinet using an airbrush. Following inoculation, the procedures for misting and growth of the plants were as described in Chapter 2. I determined the phenotype of the wildtype and mutants after 16 days using the Stakman et al. (1962) infection type scale.

4.5.2 Quantification of fungal biomass.

To determine fungal biomass in infected leaves I used the wheat germ agglutinin chitin (WAC) assay described by (Ayliffe et al. 2013) to measure chitin fluorescence. *Pgt* wildtype, *AvrSr43* mutants E1 and E7-1 were inoculated onto wheat seedlings as described above and leaf samples were harvested at 1, 2, 6 and 16 dpi. I made the following modifications to the method of Ayliffe et al (2013): harvested leaf samples were cleared in ethanol:trichloromethane (3:1, v/v) containing 0.15% (w/v) trichloroacetic acid (Moldenhauer et al. 2006). Following clearing, the samples were washed with 50% (v/v) ethanol and then they were incubated at 90 °C in 0.5 M sodium hydroxide (Moldenhauer et al. 2006) for 1 hour. The samples were then rinsed three times in 0.1 M 7.5 M Tris HCl pH 7.5. After the final rinse, the volume of Tris HCl was adjusted to give a w/v ratio of 0.2 using the fresh mass recorded at leaf sample collection. I then ground the tissue in the buffer using a mortar and pestle and returned it to a 15 ml Falcon tube.

I pipetted seven 200 µl samples of macerated tissue per treatment into PCR strips. Each sample was mixed with 10 µl of Wheat Germ Agglutinin-Alexa Fluor™ 488 conjugate (WGA-

AF488) stock (1 mg/ml) instead of WAG-FITC. I left the tissue to stain for 40 minutes followed by two repetitions of centrifuging at 1,300 g for 5 min, pipetting off excess buffer and resuspending the pelleted tissue in 150 µl 0.1 M Tris HCl pH 7.5. I loaded the inoculated samples along with the uninfected wheat leaf tissue into a Costar® 96-well black polystyrene plate. I determined the fluorescence of the samples using a Varioskan™ LUX multimode microplate reader (ThermoFisher Scientific) with the excitation wavelength set at 485 nm and 535 nm detection. The fluorescence detection period was 1 second per well. I analysed the fluorescence data by analysis of variance as comparison of means with Fisher's least significant difference (LSD).

4.5.3 Confocal imaging.

To compare pathogen growth at the cellular level between wildtype and gain-of-virulence mutants, I inoculated the *Sr43* line with *Pgt* UK-01 and its recurrent parent, Chinese Spring with two *Pgt* UK-01 gain-of-virulence mutants, E1 and E7-1, derived from 0.075 M EMS. I prepared and inoculated seedlings as previously described. Sample collection at four timepoints, clearing, treatment with NaOH and neutralisation with 0.1 M Tris HCL pH 7.5 were conducted like the WAC assay previously described. The 2 – 4 cm leaf segments were stained for 40 minutes in the dark in 2 ml centrifuge tubes containing 2 ml staining solution with 50 µl of 1 mg/ml WGA-AF488 stock and 25 µl of 1 mg/ml propidium iodide stock (Redkar et al. 2018) and 0.1 M Trish HCl. The tissue samples were gently vacuum infiltrated thrice for 5 min each along with a regular interval of 5 min with atmospheric pressure. Following infiltration, the leaf segments were rinsed twice with 0.1 M Tris HCl and stored at 4 ^{o}C in the dark (Redkar et al. 2018).

Imaging of the samples was conducted with a Zeiss® LSM 780 laser scanning confocal microscope with settings as described in Table 4.2.

Table 4.2 Excitation and detection wavelength for fluorescent dyes.

Detection	Excitation wavelength	Detection wavelength	Laser type
WGA-AF488	488 nm	500 - 540	Argon
Propidium iodide	561 nm	580 – 630 nm	Argon

(Redkar et al. 2018)

All image processing and analysis was conducted using Fiji 2 (Schindelin et al. 2012).

4.5.4 DAB staining for reactive oxygen species detection.

I prepared 12 to 14-day-old seedlings of the 20 *Sr* lines encompassing the stem rust international differential set by sowing one 9 x 9 cm pot with five seeds per cultivar. The seedlings were grown in a controlled environment room and inoculated as described in Chapter 2.

I collected leaf segments that were between five to ten centimetres in length at the timepoint previously described for the WAC assay. Staining with DAB was conducted following the procedures outlined by Daudi and O'Brien (2012). Modifications were made as follows: five to ten-centimetre leaf segments were placed in 50 ml Falcon tubes wrapped in aluminium foil containing fresh 1 mg/ml DAB solution and were then vacuum infiltrated. The leaves were rinsed with sterile dH_20 and then bleached with ethanol by boiling at 90 ^oC. The rinsed leaves were kept in an ethanol:acetic acid (3:1) mixture. The leaves were hydrated with 0.1 M Tris HCl pH 7.5 before photography.

5 Chapter 5. Conclusions and general discussion.

5.1 The main findings of this thesis:

1. The *Pgt* genome is amenable to EMS mutagenesis and my study reports a method to create mutant *Pgt* libraries for use in forward genetic screens as demonstrated by the selection and verification of multiple stable and independently derived gain-of-virulence mutants to identify Avrs.

    a. A minimum number of five gain-of-virulence mutants are predicted to be sufficient to identify causative mutations and clone a candidate *Pgt Avr.*
    b. EMS mutagenesis is effective in knocking out *Avr* genes as shown by gain-of-virulence towards *Sr43* and *Sr45* in the isolate *Pgt* UK-01.

2. Based on cytological and histological analysis of *AvrSr43* mutants infecting wheat carrying *Sr43* and its recurrent parent Chinese Spring:

    a. Loss of the *AvrSr43* has no apparent effect on pathogen fitness and this has implications for the durability of the resistance conferred by *Sr43*.
    b. *Sr43* reduces wildtype *Pgt* UK-01 pustule size, however, the number of the invasion structures is not different from that of the wildtype *Pgt.*
    c. Recognition of AvrSr43 occurs around three days post infection.

3. The development of multiple independently derived gain-of-virulence mutants against *Sr43* and *Sr45* mutants opens the opportunity to identify the corresponding *Avr* genes.

5.2 Discussion

The historical studies on the EMS mutagenesis of *Pga* and *Pgt* that led to the identification of gain-of-virulence mutants have recently inspired attempts to reproduce their results in rusts (Teo and Baker 1966; Luig 1978). The advent of next-generation sequencing technologies offers the opportunity to analyze mutant genome data leading to the identification of

candidate *Avrs*. However, one of the limiting steps has been the low success in generating *bona fide* virulent mutants. Attempts in generating virulent mutants in *Pgt* by the Steffenson group in Minnesota (personal communication) were unsuccessful. They recovered what they thought was a virulent isolate, but upon differential analysis, they determined that this was a contaminant of an isolate that was used by a colleague in a greenhouse compartment next to where they did their mutant screens. As discussed earlier, the multiple *Pst* "mutants" with multiple virulence profiles were likely contaminants too (Y. Li et al. 2019). This highlights the importance of working in an environment where there are no sources of contamination by isolates with other virulence profiles which can result in false positives. This was the clear advantage of my study due to the lack of prevailing *Pgt* isolates in the UK and the absence of groups working on exotic isolates nearby my experiments. The lack of virulent mutants from the study by the Steffenson group might also point to different responses to EMS mutagenesis by various isolates since I used their EMS mutagenesis protocol on *Pgt* UK-01. It is easier to mutate an *Avr* corresponding to an *Sr* if the *effector* locus is heterozygous. This is a probable explanation for why I could not obtain virulence towards *Sr44*. However, natural isolates with virulence towards *Sr44* do exist and these could be used in a GWAS study or sequence comparison with the avirulent parental isolate as was accomplished in the identification of AvrSr50 by comparing the virulent *Pgt*632 with avirulent *Pgt*279. Singh et al., (2013) also attempted to generate virulent mutants towards *Sr21* and *Sr35* but did not report any virulent mutants.

The first study in more recent times to successfully generate multiple independently derived EMS gain-of-virulence of *Pgt* mutants was conducted by Salcedo et al., (2017) using an isolate from the race group RKQQC. Through this work, they obtained 15 variants with common virulence on *Sr35.* These mutants maintained the same virulence profile on the stem rust international differential set and the wildtype. In this study, I obtained nine and fourteen EMS derived mutants from the isolate *Pgt* UK-01, race TKTTF, that are virulent towards *Sr43* and *S45* respectively (Kangara et al. 2020). The SNV rates obtained in the two studies were comparable at 23,010 for non-redundant SNVs for *Pgt* UK-01 and 30,429 for RKQQC (Salcedo et al. 2017; Kangara et al. 2020). The higher SNV rate might be due to longer exposure of RKQQC to EMS for 2 hours compared to 1 h 40 min in this study. The main difference with the study by Salcedo et al., (2017) is that I generated libraries of mutant *Pgt* by first inoculating

spores onto the susceptible Chinese Spring directly after EMS mutagenesis. This allowed for multiplication of over 12,000 individual mutants followed by a screening of the same population against three *Sr* genes. Moreover, the mutants could be traced back to the treatment they were derived from thereby providing information on which EMS treatments were significant in inducing gain-of-virulence. Sequencing and analysis of the 15 mutants obtained by Salcedo et al., (2017) enabled the identification of *AvSr35* and its interaction with *Sr35* was confirmed. The *AvrSr43* and *AvrSr45* mutants were sequenced and analysis is currently underway to identify the *Avrs.*

In the *Pst* EMS mutagenesis study, 33 'mutants' were derived from PSTv-18, which is avirulent to all the 18 *Yr* single-gene *Pst* differential lines. These 'mutants' were classified into 24 Pst race groups (Y. Li et al. 2019). Some of the isolates identified in the research conducted at Washington State University had race profiles like those of *Pst* isolates identified in the Washington State University's Extension resources *Pst* monitoring programme (Wan and Chen 2014; WSU Extension Resource 2020). For example, race PSTv-4 (virulence to *Yr1*, *Yr6*, *Yr9*, *Yr17*, *Yr27*, *YrSP*, *Yr76*) was first identified in 2010 and was present in 2019, whilst race PSTv-11 (virulence to *Yr1*, *Yr6*, *Yr7*, *Yr8*, *Yr9*, *Yr17*, *Yr27*, *Yr43*, *Yr44*, *YrExp2* and *Yr76*) which was first recorded in 2007 occurred in 2016 and 2017. These races, however, had a small prevalence (1.35% and 0.46%) respectively (Wan and Chen 2014; WSU Extension Resource 2020). The 33 samples had very high SNV densities between 9,353 and 117,035 per isolate and an average of 67,913 (Y. Li et al. 2020). These isolates had an almost similarly high rate of indels which is not typical of EMS mutagenesis. This is despite a short EMS exposure time of 4 to 8 min at 0.02 M or 0.03 M whilst 1h 40 min and 2 h and higher EMS concentrations were used for *Pgt* (Salcedo et al. 2017; Kangara et al. 2020). The rationale to apply GWAS on the supposedly EMS-generated population might have been motivated by the diversity of virulence of the mutants. However, this was not a segregating population.

5.3 Future applications of effector biology in plant disease control.

The study of plant-pathogen interactions has developed from the use of model crops like Arabidopsis, where important early discoveries were made, to crop plants and their economically important pathogens. This is due to the availability of cost-effective

technologies to either reduce the complexity of crop polyploid genomes (such as sequence capture or chromosome flow sorting) or to analyse them in their entirety as well as at a population level (Nordström et al. 2013; Witek et al. 2016; Arora et al. 2019; Lin et al. 2020). Through these and other approaches, several *R* genes have been identified and cloned. However, the cloning of the corresponding effectors has lagged. Several attempts that have been made to clone effectors or *Avrs* have not been successful. This is especially so for rust fungi due to limited information on rust effector features to enable better genomic prediction. Features that have been used in effector prediction are detailed in Chapter 1. This was also combined with issues to do with fragmentation of rust fungal genome assemblies generated using next-generation sequencing technologies. Where candidates have been identified, reliable methods to validate genetic interaction of effector candidates with *Sr* genes are required. More effectors will be cloned with the advent of highly accurate, affordable long-read sequencing technologies which require low DNA input such as PacBio HiFi sequencing. Togther with ONT MinION, if improved accuracy is improved, high-quality genome assemblies can be generated thus increasing success of effector identification pipelines.

Genomic prediction pipelines can identify hundreds of effector gene candidates. These then require reliable methods to screen multiple effectors on resistant lines of interest to identify *Avr-R* interactions. The development of high throughput methods for validation of candidate effectors within the wheat host could therefore lead to an increase in the number of cloned effectors for *Pgt*, *Pst*, *Pt*, and other important crop pathogens. The cloning of several effectors could lead to the identification of patterns of *Avr* structure paving the way for Machine Learning-based approaches to mine genomes for more effectors and possibly in the future, predict effector function, targets, or corresponding R genes.

R genes have been critical in identifying *Avrs*. Similarly, *Avrs* can be used to screen germplasm for their corresponding *R* genes. The effector SCR74 was used to screen the wild species *Solanum microdontum* leading to the identification of an RLK (Lin et al. 2020). This can allow for the identification of receptors that might not be present within breeding material that recognise effectors within agriculturally important pathogens. Most cloned R genes are in the NLR class; this pattern has helped to facilitate their cloning. In contrast, most *Avr* genes which

have been genetically defined and subsequently cloned have turned out to have little or no homology to previously cloned genes. This has imposed a limiting factor in the cloning of *Avrs*.

Pathogen surveillance has evolved from field pathogenomics to real-time methods such as MARPLE which allow for onsite sequencing and genotyping of pathogen isolates (Bueno-Sancho et al. 2017; Radhakrishnan et al. 2019). With increased knowledge of the effector structure or complement of rusts, these could be determined too for the prevailing isolates in a region. As such, *R* gene stacks corresponding to the effectors in the pathogen population could then be deployed. Eventually, it will be possible to determine (virulence) phenotype from genotype alone.

Besides the use of resistance receptors for pathogen control, HIGS can be employed to silence effectors involved in virulence since plants can deliver siRNAs into fungi. Also, identification of virulence targets and modifying them to reduce susceptibility as was accomplished in the CRISPR/Cas9 editing of the *Mlo* allele *SlMlo1* in tomato making it resistant to mildew (Nekrasov et al. 2017). Modification of NLR integrated domains has been shown to expand effector recognition (De la Concepcion et al. 2018). Recent effector identification studies have revealed that effectors are not comprised of proteins only e.g. sRNAs (Wang et al. 2017; Trutzenberg, et al. 2020). These need to be identified as they play significant roles in plant-pathogen interactions.

With the world population expected to double by 2050, agricultural production is projected to need to increase by between 60% and 110% to meet the demand for food (Godfray et al. 2010). However, genetic gain from wheat breeding programmes is currently at 1% (Tadesse et al. 2019) against a minimum required forecast rate of 2.4% (Ray et al. 2013). Diseases in wheat account for up to 28% losses (Savary et al. 2019b). The application of the rapidly increasing findings from plant-pathogen interactions studies is beginning to yield fruit in the control of diseases affecting several economically important crops. The reduction of crop losses due to diseases could significantly contribute to food security through the preservation of potential yield.

6. Chapter 6. References

2Blades Foundation. 2019. “Wheat Lines from 2Blades, CSIRO, and UMN Exhibit Exceptional Stem Rust Resistance in the Field.” 2019. https://2blades.org/2019/03/12/wheat-lines-from-2blades-csiro-and-umn-exhibit-exceptional-stem-rust-resistance-in-the-field/.

Abdullah, Araz S., Chala Turo, Caroline S. Moffat, Francisco J. Lopez-Ruiz, Mark R. Gibberd, John Hamblin, and Ayalsew Zerihun. 2018. “Real-Time PCR for Diagnosing and Quantifying Co-Infection by Two Globally Distributed Fungal Pathogens of Wheat.” *Frontiers in Plant Science* 0 (August): 1086. https://doi.org/10.3389/FPLS.2018.01086.

Adachi, Hiroaki, Lida Derevnina, and Sophien Kamoun. 2019. “NLR Singletons, Pairs, and Networks: Evolution, Assembly, and Regulation of the Intracellular Immunoreceptor Circuitry of Plants.” *Current Opinion in Plant Biology* 50 (August): 121–31. https://doi.org/10.1016/j.pbi.2019.04.007.

Addo-Quaye, Charles, Elizabeth Buescher, Norman Best, Vijay Chaikam, Ivan Baxter, and Brian P. Dilkes. 2017. “Forward Genetics by Sequencing EMS Variation-Induced Inbred Lines.” *G3: Genes, Genomes, Genetics* 7 (2): 413–25. https://doi.org/10.1534/g3.116.029660.

Agrios, George. 2005. *Plant Pathology*. Edited by Dana Dreibelbis. Fifth. Vol. 231. San Diego: Elsevier Academic Press. https://doi.org/10.1002/1521-3773(20010316)40:6<9823::AID-ANIE9823>3.3.CO;2-C.

AHDB. 2020. “Brown Rust in Cereals | AHDB.” 2020. https://ahdb.org.uk/brownrust.

Ahmed, Ali Abdurehim, Carsten Pedersen, Torsten Schultz-Larsen, Mark Kwaaitaal, Hans Jørgen Lyngs Jørgensen, and Hans Thordal-Christensen. 2015. “The Barley Powdery Mildew Candidate Secreted Effector Protein CSEP0105 Inhibits the Chaperone Activity of a Small Heat Shock Protein.” *Plant Physiology* 168 (1): 321–33. https://doi.org/10.1104/pp.15.00278.

Ahmed, Hadia, T. C. Howton, Yali Sun, Natascha Weinberger, Youssef Belkhadir, and M. Shahid Mukhtar. 2018. “Network Biology Discovers Pathogen Contact Points in Host Protein-Protein Interactomes.” *Nature Communications* 9 (1): 1–13. https://doi.org/10.1038/s41467-018-04632-8.

Ahmed, Md Bulbul, Karen Cristine Gonçalves dos Santos, Ingrid Benerice Sanchez, Benjamin Petre, Cécile Lorrain, Mélodie B. Plourde, Sébastien Duplessis, Isabel Desgagné-Penix,

and Hugo Germain. 2018. “A Rust Fungal Effector Binds Plant DNA and Modulates Transcription.” *Scientific Reports* 8 (1): 14718. https://doi.org/10.1038/s41598-018-32825-0.

Al-Adil, K. M., and R. F. Al-Jassany. 1992. “A Preliminary Study of the Relationship between Mixing Corn Varieties and the Susceptibility to Infestation with the Corn Stem Borer, Sesamia Crerica (Lepidoptera: Noctuidae).” *Arab Journal of Plant Protection* 10: 232–38.

Albert, Markus, Anna K. Jehle, Martin Lipschis, Katharina Mueller, Yi Zeng, and Georg Felix. 2010. “Regulation of Cell Behaviour by Plant Receptor Kinases: Pattern Recognition Receptors as Prototypical Models.” *European Journal of Cell Biology* 89 (2–3): 200–207. https://doi.org/10.1016/j.ejcb.2009.11.015.

Anand, Ajith, Harold N. Trick, Bikram S. Gill, and Subbaratnam Muthukrishnan. 2003. “Stable Transgene Expression and Random Gene Silencing in Wheat.” *Plant Biotechnology Journal* 1 (4): 241–51. https://doi.org/10.1046/j.1467-7652.2003.00023.x.

Andersen, Ethan J., Madhav P. Nepal, Jordan M. Purintun, Dillon Nelson, Glykeria Mermigka, and Panagiotis F. Sarris. 2020. “Wheat Disease Resistance Genes and Their Diversification Through Integrated Domain Fusions.” *Frontiers in Genetics* 11 (August): 898. https://doi.org/10.3389/fgene.2020.00898.

Anderson, Claire, Muhammad Adil Khan, Ann-Maree Catanzariti, Cameron A. Jack, Adnane Nemri, Gregory J. Lawrence, Narayana M. Upadhyaya, et al. 2016. “Genome Analysis and Avirulence Gene Cloning Using a High-Density RADseq Linkage Map of the Flax Rust Fungus, Melampsora Lini.” *BMC Genomics* 17 (1): 667. https://doi.org/10.1186/s12864-016-3011-9.

Andolfo, Giuseppe, Antimo Di Donato, Pasquale Chiaiese, Antonino De Natale, Antonino Pollio, Jonathan D.G. Jones, Luigi Frusciante, and Maria Raffaella Ercolano. 2019. “Alien Domains Shaped the Modular Structure of Plant NLR Proteins.” *Genome Biology and Evolution* 11 (12): 3466–77. https://doi.org/10.1093/gbe/evz248.

Arena, Maria, Domenica Auteri, Stefania Barmaz, Giulia Bellisai, Alba Brancato, Daniela Brocca, Laszlo Bura, et al. 2018. “Peer Review of the Pesticide Risk Assessment of the Active Substance Chlorothalonil.” *EFSA Journal* 16 (1). https://doi.org/10.2903/j.efsa.2018.5126.

Armstrong, Miles R., Stephen C. Whisson, Leighton Pritchard, Jorunn I.B. Bos, Eduard

Venter, Anna O. Avrova, Anne P. Rehmany, et al. 2005. “An Ancestral Oomycete Locus Contains Late Blight Avirulence Gene Avr3a, Encoding a Protein That Is Recognized in the Host Cytoplasm.” *Proceedings of the National Academy of Sciences of the United States of America* 102 (21): 7766–71. https://doi.org/10.1073/pnas.0500113102.

Arora, Sanu, Burkhard Steuernagel, Kumar Gaurav, Sutha Chandramohan, Yunming Long, Oadi Matny, Ryan Johnson, et al. 2019. “Resistance Gene Cloning from a Wild Crop Relative by Sequence Capture and Association Genetics.” *Nature Biotechnology* 37 (2): 139–43. https://doi.org/10.1038/s41587-018-0007-9.

Artz, Derek R., and Theresa L. Pitts-Singer. 2015. “Effects of Fungicide and Adjuvant Sprays on Nesting Behavior in Two Managed Solitary Bees, Osmia Lignaria and Megachile Rotundata.” Edited by Nicolas Desneux. *PLoS ONE* 10 (8): e0135688. https://doi.org/10.1371/journal.pone.0135688.

Ashelford, Kevin, Maria E. Eriksson, Christopher M. Allen, Rosalinda D’Amore, Mikael Johansson, Peter Gould, Suzanne Kay, Andrew J. Millar, Neil Hall, and Anthony Hall. 2011. “Full Genome Re-Sequencing Reveals a Novel Circadian Clock Mutation in Arabidopsis.” *Genome Biology* 12 (3): R28. https://doi.org/10.1186/gb-2011-12-3-r28.

Axtell, Michael J., and Brian J. Staskawicz. 2003. “Initiation of RPS2-Specified Disease Resistance in Arabidopsis Is Coupled to the AvrRpt2-Directed Elimination of RIN4.” *Cell* 112 (3): 369–77. https://doi.org/10.1016/S0092-8674(03)00036-9.

Ayliffe, Michael, Sambasivam K Periyannan, Angela Feechan, Ian Dry, Ulrike Schumann, Ming Bo Wang, Anthony Pryor, and Evans Lagudah. 2013. “A Simple Method for Comparing Fungal Biomass in Infected Plant Tissues.” *Molecular Plant-Microbe Interactions* 26 (6): 658–67. https://doi.org/10.1094/MPMI-12-12-0291-R.

Ayliffe, Michael, and Chris K. Sørensen. 2019. “Plant Nonhost Resistance: Paradigms and New Environments.” *Current Opinion in Plant Biology*. Elsevier Ltd. https://doi.org/10.1016/j.pbi.2019.03.011.

Baggs, E, G Dagdas, and K. V. Krasileva. 2017. “NLR Diversity, Helpers and Integrated Domains: Making Sense of the NLR IDentity.” *Current Opinion in Plant Biology*. https://doi.org/10.1016/j.pbi.2017.04.012.

Baird, Duncan M. 2018. “Telomeres and Genomic Evolution.” *Philosophical Transactions of the Royal Society B: Biological Sciences*. Royal Society Publishing. https://doi.org/10.1098/rstb.2016.0437.

Balesdent, Marie-Hélène, CléMenCe Plissonneau, Laurent Coudard, GuillauMe Daverdin, LoïC le Meur, Julien CarPezat, Martine Leflon, et al. 2015. “Résistance Du Colza Au Phoma : Où En Est l’efficacité de Rlm7 ? De Nombreuses Parcelles de Colza Bénéficient de La Résistance Variétale Au Phoma Apportée Par Le Gène Rlm7. Cela va-t-Il Durer ?” *Phytoma* 684.

Balesdent, Marie H??l??ne, Isabelle Fudal, B??n??dicte Ollivier, Pascal Bally, Jonathan Grandaubert, Fr??d??rique Eber, Anne Marie Ch??vre, Martine Leflon, and Thierry Rouxel. 2013. “The Dispensable Chromosome of Leptosphaeria Maculans Shelters an Effector Gene Conferring Avirulence towards Brassica Rapa.” *New Phytologist* 198 (3): 887–98. https://doi.org/10.1111/nph.12178.

Baltrus, David A., Marc T. Nishimura, Artur Romanchuk, Jeff H. Chang, M. Shahid Mukhtar, Karen Cherkis, Jeff Roach, Sarah R. Grant, Corbin D. Jones, and Jeffery L. Dangl. 2011. “Dynamic Evolution of Pathogenicity Revealed by Sequencing and Comparative Genomics of 19 Pseudomonas Syringae Isolates.” Edited by David S. Guttman. *PLoS Pathogens* 7 (7): e1002132. https://doi.org/10.1371/journal.ppat.1002132.

Barnes, Gerry, Diane G. O. Saunders, and Tom Williamson. 2020. “Banishing Barberry: The History of *Berberis Vulgaris* Prevalence and Wheat Stem Rust Incidence across Britain.” *Plant Pathology* 69 (7): 1193–1202. https://doi.org/10.1111/ppa.13231.

Barragan, A Cristina, Maximilian Collenberg, Jinge Wang, Rachelle R Q Lee, Wei Yuan Cher, Fernando A Rabanal, Haim Ashkenazy, Detlef Weigel, and Eunyoung Chae. 2020. “A Truncated Singleton NLR Causes Hybrid Necrosis in Arabidopsis Thaliana.” Edited by Patricia Wittkopp. *Molecular Biology and Evolution*, September. https://doi.org/10.1093/molbev/msaa245.

Bayer Crop Science. 2020. “Folicur.” 2020. https://cropscience.bayer.co.uk/our-products/fungicides/folicur/.

Beddow, Jason M, Terry M Hurley, Darren J Kriticos, and Philip G Pardey. 2013. “Measuring the Global Occurrence and Probabilistic Consequences of Wheat Stem Rust.” *HarvestChoice Technical Note*, no. c: 23.

Bekal, Sadia, Terry L. Niblack, and Kris N. Lambert. 2003. “A Chorismate Mutase from the Soybean Cyst Nematode Heterodera Glycines Shows Polymorphisms That Correlate with Virulence.” *Molecular Plant-Microbe Interactions* 16 (5): 439–46. https://doi.org/10.1094/MPMI.2003.16.5.439.

Bentham, Adam, Hayden Burdett, Peter A. Anderson, Simon J. Williams, and Bostjan Kobe. 2017. “Animal NLRs Provide Structural Insights into Plant NLR Function.” *Annals of Botany*. Oxford University Press. https://doi.org/10.1093/aob/mcw171.

Bentham, Adam R, Juan Carlos De la Concepcion, Nitika Mukhi, Rafał Zdrzałek, Markus Draeger, Danylo Gorenkin, Richard K. Hughes, and Mark J. Banfield. 2020. “A Molecular Roadmap to the Plant Immune System.” *Journal of Biological Chemistry*, August, jbc.REV120.010852. https://doi.org/10.1074/jbc.REV120.010852.

Berlin, Anna, Berit Samils, and Björn Andersson. 2017. “Multiple Genotypes within Aecial Clusters in Puccinia Graminis and Puccinia Coronata: Improved Understanding of the Biology of Cereal Rust Fungi.” *Fungal Biology and Biotechnology* 4 (1): 3. https://doi.org/10.1186/s40694-017-0032-3.

BGRI. n.d. “About BGRI.” Accessed August 25, 2020. https://www.globalrust.org/about-bgri.

Białas, Aleksandra, Erin K. Zess, Juan Carlos De la Concepcion, Marina Franceschetti, Helen G. Pennington, Kentaro Yoshida, Jessica L. Upson, et al. 2018. “Lessons in Effector and NLR Biology of Plant-Microbe Systems.” *Molecular Plant-Microbe Interactions®* 31 (1): 34–45. https://doi.org/10.1094/MPMI-08-17-0196-FI.

Białas, Aleksandra, Erin K Zess, Juan Carlos De La Concepcion, Marina Franceschetti, Helen G Pennington, Kentaro Yoshida, Jessica L Upson, et al. n.d. “Lessons in Effector and NLR Biology of Plant-Microbe Systems.” Accessed August 27, 2017. https://doi.org/10.1101/171223.

Biezen, Erik A. Van Der, and Jonathan D.G. Jones. 1998. “Plant Disease-Resistance Proteins and the Gene-for-Gene Concept.” *Trends in Biochemical Sciences* 23 (12): 454–56. https://doi.org/10.1016/S0968-0004(98)01311-5.

Blondeau, Karine, Françoise Blaise, Marc Graille, Shiv D. Kale, Juliette Linglin, Bénédicte Ollivier, Audrey Labarde, et al. 2015. “Crystal Structure of the Effector AvrLm4-7 of *Leptosphaeria Maculans* Reveals Insights into Its Translocation into Plant Cells and Recognition by Resistance Proteins.” *The Plant Journal* 83 (4): 610–24. https://doi.org/10.1111/tpj.12913.

Bockhaven, Jonas Van, Lukáš Spíchal, Ondřej Novák, Miroslav Strnad, Takayuki Asano, Shoshi Kikuchi, Monica Höfte, and David De Vleesschauwer. 2015. “Silicon Induces Resistance to the Brown Spot Fungus Cochliobolus Miyabeanus by Preventing the Pathogen from Hijacking the Rice Ethylene Pathway.” *New Phytologist* 206 (2): 761–73.

https://doi.org/10.1111/nph.13270.

Bockus, William W, Jon A Appel, Robert L Bowden, Allan K Fritz, Bikram S Gill, T. Joe Martin, Rollin G Sears, Dallas L Seifers, Gina L Brown-Guedira, and Merle G Eversmeyer. 2001. "Success Stories: Breeding for Wheat Disease Resistance in Kansas." *Plant Disease* 85 (5): 453–61. https://doi.org/10.1094/PDIS.2001.85.5.453.

Böhnert, Heidi U., Isabelle Fudal, Waly Dioh, Didier Tharreau, Jean Loup Notteghem, and Marc Henri Lebrun. 2004. "A Putative Polyketide Synthase/Peptide Synthetase from Magnaporthe Grisea Signals Pathogen Attack to Resistant Rice." *Plant Cell* 16 (9): 2499–2513. https://doi.org/10.1105/tpc.104.022715.

Boller, Thomas, and Georg Felix. 2009. "A Renaissance of Elicitors: Perception of Microbe-Associated Molecular Patterns and Danger Signals by Pattern-Recognition Receptors." *Annual Review of Plant Biology* 60 (1): 379–406. https://doi.org/10.1146/annurev.arplant.57.032905.105346.

Bolton, Melvin D., H. Peter Van Esse, Jack H. Vossen, Ronnie De Jonge, Ioannis Stergiopoulos, Iris J.E. Stulemeijer, Grardy C.M. Van Den Berg, et al. 2008. "The Novel Cladosporium Fulvum Lysin Motif Effector Ecp6 Is a Virulence Factor with Orthologues in Other Fungal Species." *Molecular Microbiology* 69 (1): 119–36. https://doi.org/10.1111/j.1365-2958.2008.06270.x.

Bonardi, Vera, Saijun Tang, Anna Stallmann, Melinda Roberts, Karen Cherkis, and Jeffery L. Dangl. 2011. "Expanded Functions for a Family of Plant Intracellular Immune Receptors beyond Specific Recognition of Pathogen Effectors." *Proceedings of the National Academy of Sciences of the United States of America* 108 (39): 16463–68. https://doi.org/10.1073/pnas.1113726108.

Bourras, Salim, Kaitlin Elyse McNally, Roi Ben-David, Francis Parlange, Stefan Roffler, Coraline Rosalie Praz, Simone Oberhaensli, et al. 2015. "Multiple Avirulence Loci and Allele-Specific Effector Recognition Control the Pm3 Race-Specific Resistance of Wheat to Powdery Mildewopen." *Plant Cell* 27 (10): 2991–3012. https://doi.org/10.1105/tpc.15.00171.

Bouton, Clément, Robert C. King, Hongxin Chen, Kasi Azhakanandam, Stéphane Bieri, Kim E. Hammond-Kosack, and Kostya Kanyuka. 2018. "Foxtail Mosaic Virus: A Viral Vector for Protein Expression in Cereals." *Plant Physiology* 177 (4): 1352–67. https://doi.org/10.1104/pp.17.01679.

Boutrot, Freddy, and Cyril Zipfel. 2017. “Function, Discovery, and Exploitation of Plant Pattern Recognition Receptors for Broad-Spectrum Disease Resistance.” *Annual Review of Phytopathology*. https://doi.org/10.1146/annurev-phyto-080614-120106.

Breiden, Maike, and Rudiger Simon. 2016. “Q&A: How Does Peptide Signaling Direct Plant Development?” *BMC Biology* 14 (1): 58. https://doi.org/10.1186/s12915-016-0280-3.

Broad Institute. 2009. “Picard Tools - By Broad Institute.” Github. 2009. http://broadinstitute.github.io/picard/.

Browder, L E. 1971. “Pathogenic Specialization in Cereal Rust Fungi, Especially Puccinia Recondita f. Sp. Tritici: Concepts, Methods of Study, and Application.” Washington DC.

Brown, J. K M, and J. C. Rant. 2013. “Fitness Costs and Trade-Offs of Disease Resistance and Their Consequences for Breeding Arable Crops.” *Plant Pathology* 62 (S1): 83–95. https://doi.org/10.1111/ppa.12163.

Brown, James K.M., and Mogens S. Hovmøll. 2002. “Aerial Dispersal of Pathogens on the Global and Continental Scales and Its Impact on Plant Disease.” *Science*. American Association for the Advancement of Science. https://doi.org/10.1126/science.1072678.

Brueggeman, R., A. Druka, J. Nirmala, T. Cavileer, T. Drader, N. Rostoks, A. Mirlohi, et al. 2008. “The Stem Rust Resistance Gene Rpg5 Encodes a Protein with Nucleotide-Binding-Site, Leucine-Rich, and Protein Kinase Domains.” *Proceedings of the National Academy of Sciences of the United States of America* 105 (39): 14970–75. https://doi.org/10.1073/pnas.0807270105.

Brueggeman, R., N. Rostoks, D. Kudrna, A. Kilian, F. Han, J. Chen, A. Druka, B. Steffenson, and A. Kleinhofs. 2002. “The Barley Stem Rust-Resistance Gene Rpg1 Is a Novel Disease-Resistance Gene with Homology to Receptor Kinases.” *Proceedings of the National Academy of Sciences of the United States of America* 99 (14): 9328–33. https://doi.org/10.1073/pnas.142284999.

Bueno-Sancho, Vanessa, Daniel C. E. Bunting, Luis J. Yanes, Kentaro Yoshida, and Diane G. O. Saunders. 2017. “Field Pathogenomics: An Advanced Tool for Wheat Rust Surveillance.” In , 13–28. https://doi.org/10.1007/978-1-4939-7249-4_2.

Burdon, J J, and J Silk. 1997. “Sources and Patterns of Diversity in Plant-Pathogenic Fungi.” *Phytopathology* 87 (7): 664–69. https://doi.org/10.1094/PHYTO.1997.87.7.664.

Burg, Harrold A. Van Den, Stuart J. Harrison, Matthieu H.A.J. Joosten, Jacques Vervoort, and Pierre J.G.M. De Wit. 2006. “Cladosporium Fulvum Avr4 Protects Fungal Cell Walls

against Hydrolysis by Plant Chitinases Accumulating during Infection." *Molecular Plant-Microbe Interactions* 19 (12): 1420–30. https://doi.org/10.1094/MPMI-19-1420.

Bushnell, William. 1984. *The Cereal Rusts : Origins, Specificity, Structure and Physiology.* Edited by William R. Bushnell and Alan P. Roelfs. *The Cereal Rusts: Origins, Specificity, Structure, and …*. Vol. 1. Orlando: Academic Press Inc. https://www.ars.usda.gov/ARSUserFiles/50620500/Cerealrusts/The Cereal Rusts_VOLUME I.pdf#page=491%0Ahttp://books.google.com/books?hl=en&lr=&id=jIClNPdFDMUC&oi=fnd&pg=PA131&dq=race+specificity+and+methods+of+study&ots=EXvdE9VAU7&sig=TR38KH9tKbsDO6hDiFBsemF.

Büttner, Daniela. 2016. "Behind the Lines-Actions of Bacterial Type III Effector Proteins in Plant Cells." *FEMS Microbiology Reviews*. Oxford University Press. https://doi.org/10.1093/femsre/fuw026.

Cai, Qiang, Lulu Qiao, Ming Wang, Baoye He, Feng Mao Lin, Jared Palmquist, Sienna Da Huang, and Hailing Jin. 2018. "Plants Send Small RNAs in Extracellular Vesicles to Fungal Pathogen to Silence Virulence Genes." *Science* 360 (6393): 1126–29. https://doi.org/10.1126/science.aar4142.

Cai, Rongman, James Lewis, Shuangchun Yan, Haijie Liu, Christopher R. Clarke, Francesco Campanile, Nalvo F. Almeida, et al. 2011. "The Plant Pathogen Pseudomonas Syringae Pv. Tomato Is Genetically Monomorphic and under Strong Selection to Evade Tomato Immunity." Edited by David S. Guttman. *PLoS Pathogens* 7 (8): e1002130. https://doi.org/10.1371/journal.ppat.1002130.

Caicedo, Ana L., and Barbara A. Schaal. 2004. "Heterogeneous Evolutionary Process Affect R Gene Diversity in Natural Populations of Solanum Pimpinellifolium." *Proceedings of the National Academy of Sciences of the United States of America* 101 (50): 17444–49. https://doi.org/10.1073/pnas.0407899101.

Cantu, D, V Segovia, D MacLean, R Bayles, X Chen, S Kamoun, J Dubcovsky, D G Saunders, and C Uauy. 2013. "Genome Analyses of the Wheat Yellow (Stripe) Rust Pathogen Puccinia Striiformis f. Sp. Tritici Reveal Polymorphic and Haustorial Expressed Secreted Proteins as Candidate Effectors." *BMC Genomics* 14: 270. https://doi.org/10.1186/1471-2164-14-270\n1471-2164-14-270 [pii].

Cao, Lingxue, Mila C. Blekemolen, Nico Tintor, Ben J.C. Cornelissen, and Frank L.W. Takken.

2018. “The Fusarium Oxysporum Avr2-Six5 Effector Pair Alters Plasmodesmatal Exclusion Selectivity to Facilitate Cell-to-Cell Movement of Avr2.” *Molecular Plant* 11 (5): 691–705. https://doi.org/10.1016/j.molp.2018.02.011.

Caplan, Jeffrey L., Padmavathi Mamillapalli, Tessa M. Burch-Smith, Kirk Czymmek, and S. P. Dinesh-Kumar. 2008. “Chloroplastic Protein NRIP1 Mediates Innate Immune Receptor Recognition of a Viral Effector.” *Cell* 132 (3): 449–62. https://doi.org/10.1016/j.cell.2007.12.031.

Castro, A. 2001. “Cultivar Mixtures.” *The Plant Health Instructor*. https://doi.org/10.1094/phi-a-2001-1230-01.

Catanzariti, AM, PN Dodds, GJ Lawrence, MA Ayliffe, and JG Ellis. 2006. “Haustorially Expressed Secreted Proteins from Flax Rust Are Highly Enriched for Avirulence Elicitors.” *Plant Cell* 18.

Catanzariti, Ann Maree, Peter N. Dodds, Gregory J. Lawrence, Michael A. Ayliffe, and Jeffrey G. Ellis. 2006. “Haustorially Expressed Secreted Proteins from Flax Rust Are Highly Enriched for Avirulence Elicitors.” *Plant Cell* 18 (1): 243–56. https://doi.org/10.1105/tpc.105.035980.

Cesari, Stella, Maud Bernoux, Philippe Moncuquet, Thomas Kroj, and Peter N. Dodds. 2014. “A Novel Conserved Mechanism for Plant NLR Protein Pairs: The Â€œintegrated Decoyâ€ Hypothesis.” *Frontiers in Plant Science* 5 (NOV): 25. https://doi.org/10.3389/fpls.2014.00606.

Césari, Stella, Hiroyuki Kanzaki, Tadashi Fujiwara, Maud Bernoux, Véronique Chalvon, Yoji Kawano, Ko Shimamoto, Peter Dodds, Ryohei Terauchi, and Thomas Kroj. 2014. “ The NB - LRR Proteins RGA 4 and RGA 5 Interact Functionally and Physically to Confer Disease Resistance .” *The EMBO Journal* 33 (17): 1941–59. https://doi.org/10.15252/embj.201487923.

Cesari, Stella, Gaëtan Thilliez, Cécile Ribot, Véronique Chalvon, Corinne Michel, Alain Jauneau, Susana Rivas, et al. 2013a. “The Rice Resistance Protein Pair RGA4/RGA5 Recognizes the *Magnaporthe Oryzae* Effectors AVR-Pia and AVR1-CO39 by Direct Binding.” *The Plant Cell* 25 (4). https://doi.org/10.1105/tpc.112.107201.

———. 2013b. “The Rice Resistance Protein Pair RGA4/RGA5 Recognizes the Magnaporthe Oryzae Effectors AVR-Pia and AVR1-CO39 by Direct Binding.” *Plant Cell* 25 (4): 1463–81. https://doi.org/10.1105/tpc.112.107201.

Chakraborty, S., and A. C. Newton. 2011. “Climate Change, Plant Diseases and Food Security: An Overview.” *Plant Pathology* 60 (1): 2–14. https://doi.org/10.1111/j.1365-3059.2010.02411.x.

Chauhan, R., M. Farman, H. B. Zhang, and S. Leong. 2002. “Genetic and Physical Mapping of a Rice Blast Resistance Locus, Pi-CO39(t), That Corresponds to the Avirulence Gene AVR1-CO39 of Magnaporthe Grisea.” *Molecular Genetics and Genomics* 267 (5): 603–12. https://doi.org/10.1007/s00438-002-0691-4.

Cheeke, P. R. 1998. “Natural Toxicants in Feeds, Forages, and Poisonous Plants.” *Natural Toxicants in Feeds, Forages, and Poisonous Plants.*, no. Ed. 2.

Chen, Chunhong, Matthias Jost, Bethany Clark, Matthew Martin, Oadi Matny, Brian J. Steffenson, Jerome D. Franckowiak, et al. 2021. “BED Domain-Containing NLR from Wild Barley Confers Resistance to Leaf Rust.” *Plant Biotechnology Journal* 19 (6): 1206–15. https://doi.org/10.1111/PBI.13542.

Chen, Grace Y., and Gabriel Nuñez. 2010. “Sterile Inflammation: Sensing and Reacting to Damage.” *Nature Reviews Immunology*. Nature Publishing Group. https://doi.org/10.1038/nri2873.

Chen, Jiapeng. 2017. “Genomics Studies of Two Cereal Rust Fungi with a Focus on Avirulence Gene Searches.” University of Sydney. https://ses.library.usyd.edu.au/handle/2123/17658.

Chen, Jiapeng, Narayana M Upadhyaya, Diana Ortiz, Jana Sperschneider, Feng Li, Clement Bouton, Susan Breen, et al. 2017. “Loss of AvrSr50 by Somatic Exchange in Stem Rust Leads to Virulence for Sr50 Resistance in Wheat.” *Science* 358 (6370): 1607–10. https://doi.org/10.1126/science.aao4810.

Chen, Shisheng, Matthew N. Rouse, Wenjun Zhang, Xiaoqin Zhang, Yan Guo, Jordan Briggs, and Jorge Dubcovsky. 2020. “Wheat Gene Sr60 Encodes a Protein with Two Putative Kinase Domains That Confers Resistance to Stem Rust.” *New Phytologist* 225 (2): 948–59. https://doi.org/10.1111/nph.16169.

Chen, Shisheng, Wenjun Zhang, Stephen Bolus, Matthew N. Rouse, and Jorge Dubcovsky. 2018a. “Identification and Characterization of Wheat Stem Rust Resistance Gene Sr21 Effective against the Ug99 Race Group at High Temperature.” Edited by Beat Keller. *PLoS Genetics* 14 (4): e1007287. https://doi.org/10.1371/journal.pgen.1007287.

———. 2018b. “Identification and Characterization of Wheat Stem Rust Resistance Gene

Sr21 Effective against the Ug99 Race Group at High Temperature." Edited by Beat Keller. *PLoS Genetics* 14 (4): e1007287. https://doi.org/10.1371/journal.pgen.1007287.

Chen, Songbiao, Pattavipha Songkumarn, R C Venu, Malali Gowda, Maria Bellizzi, Jinnan Hu, Wende Liu, et al. 2013. "Identification and Characterization of in Planta-Expressed Secreted Effector Proteins from Magnaporthe Oryzae That Induce Cell Death in Rice." *Molecular Plant-Microbe Interactions : MPMI* 26 (2): 191–202. https://doi.org/10.1094/MPMI-05-12-0117-R.

Cheng, Yulin, Xiaojie Wang, Juanni Yao, Ralf T. Voegele, Yanru Zhang, Wumei Wang, Lili Huang, and Zhensheng Kang. 2015. "Characterization of Protein Kinase PsSRPKL, a Novel Pathogenicity Factor in the Wheat Stripe Rust Fungus." *Environmental Microbiology* 17 (8): 2601–17. https://doi.org/10.1111/1462-2920.12719.

Cheng, Yulin, Kuan Wu, Juanni Yao, Shumin Li, Xiaojie Wang, Lili Huang, and Zhensheng Kang. 2017. "PSTha5a23, a Candidate Effector from the Obligate Biotrophic Pathogen *P Uccinia Striiformis* f. Sp. *Tritici* , Is Involved in Plant Defense Suppression and Rust Pathogenicity." *Environmental Microbiology* 19 (5): 1717–29. https://doi.org/10.1111/1462-2920.13610.

Cheng, Yulin, Juanni Yao, Yanru Zhang, Shumin Li, and Zhensheng Kang. 2016. "Characterization of a Ran Gene from Puccinia Striiformis f. Sp. Tritici Involved in Fungal Growth and Anti-Cell Death." *Scientific Reports* 6 (October). https://doi.org/10.1038/srep35248.

Chilton, M. D., R. K. Saiki, and N. Yadav. 1980. "T-DNA from Agrobacterium Ti Plasmid Is in the Nuclear DNA Fraction of Crown Gall Tumor Cells." *Proceedings of the National Academy of Sciences of the United States of America* 77 (7 II): 4060–64. https://doi.org/10.1073/pnas.77.7.4060.

Chisholm, Stephen T., Gitta Coaker, Brad Day, and Brian J. Staskawicz. 2006. "Host-Microbe Interactions: Shaping the Evolution of the Plant Immune Response." *Cell* 124 (4): 803–14. https://doi.org/10.1016/j.cell.2006.02.008.

Ciuffetti, Lynda M., Robert P. Tuori, and Janey M. Gaventa. 1997. "A Single Gene Encodes a Selective Toxin Causal to the Development of Tan Spot of Wheat." *Plant Cell* 9 (2): 135–44. https://doi.org/10.1105/tpc.9.2.135.

Cloutier, Sylvie, Brent D. McCallum, Caroline Loutre, Travis W. Banks, Thomas Wicker, Catherine Feuillet, Beat Keller, and Mark C. Jordan. 2007. "Leaf Rust Resistance Gene

Lr1, Isolated from Bread Wheat (Triticum Aestivum L.) Is a Member of the Large Psr567 Gene Family." *Plant Molecular Biology* 65 (1–2): 93–106. https://doi.org/10.1007/s11103-007-9201-8.

Coaker, Gitta, Arnold Falick, and Brian Staskawicz. 2005. "Activation of a Phytopathogenic Bacterial Effector Protein by a Eukaryotic Cyclophilin." *Science* 308 (5721): 548–50. https://doi.org/10.1126/science.1108633.

Collemare, Jérôme, Richard O'Connell, and Marc-Henri Lebrun. 2019. "Nonproteinaceous Effectors: The *Terra Incognita* of Plant–Fungal Interactions." *New Phytologist* 223 (2): 590–96. https://doi.org/10.1111/nph.15785.

Collemare, Jérôme, Mikaël Pianfetti, Anne Elodie Houlle, Damien Morin, Laurent Camborde, Marie Josèphe Gagey, Crystel Barbisan, Isabelle Fudal, Marc Henri Lebrun, and Heidi U. Böhnert. 2008. "Magnaporthe Grisea Avirulence Gene ACE1 Belongs to an Infection-Specific Gene Cluster Involved in Secondary Metabolism." *New Phytologist* 179 (1): 196–208. https://doi.org/10.1111/j.1469-8137.2008.02459.x.

Collier, Sarah M., Louis Philippe Hamel, and Peter Moffett. 2011. "Cell Death Mediated by the N-Terminal Domains of a Unique and Highly Conserved Class of NB-LRR Protein." *Molecular Plant-Microbe Interactions* 24 (8): 918–31. https://doi.org/10.1094/MPMI-03-11-0050.

Collins, N. C., C. A. Webb, S. Seah, J. G. Ellis, S. H. Hulbert, and A. Pryor. 1998. "The Isolation and Mapping of Disease Resistance Gene Analogs in Maize." *Molecular Plant-Microbe Interactions* 11 (10): 968–78. https://doi.org/10.1094/MPMI.1998.11.10.968.

Corredor-Moreno, Pilar, and Diane G.O. Saunders. 2020. "Expecting the Unexpected: Factors Influencing the Emergence of Fungal and Oomycete Plant Pathogens." *New Phytologist*. Blackwell Publishing Ltd. https://doi.org/10.1111/nph.16007.

Cristancho, Marco A., David Octavio Botero-Rozo, William Giraldo, Javier Tabima, Diego Mauricio RiaÃ±o-PachÃ³n, Carolina Escobar, Yomara Rozo, et al. 2014. "Annotation of a Hybrid Partial Genome of the Coffee Rust (Hemileia Vastatrix) Contributes to the Gene Repertoire Catalog of the Pucciniales." *Frontiers in Plant Science* 5 (October): 594. https://doi.org/10.3389/fpls.2014.00594.

Crossa, J., P. Pérez, J. Hickey, J. Burgueño, L. Ornella, J. Cerón-Rojas, X. Zhang, et al. 2014. "Genomic Prediction in CIMMYT Maize and Wheat Breeding Programs." *Heredity* 112 (1): 48–60. https://doi.org/10.1038/hdy.2013.16.

Cruz, Christian D., and Barbara Valent. 2017. “Wheat Blast Disease: Danger on the Move.” *Tropical Plant Pathology*, May. https://doi.org/10.1007/s40858-017-0159-z.

Cunnac, Sébastien, Suma Chakravarthy, Brian H. Kvitko, Alistair B. Russell, Gregory B. Martin, and Alan Collmer. 2011. “Genetic Disassembly and Combinatorial Reassembly Identify a Minimal Functional Repertoire of Type III Effectors in Pseudomonas Syringae.” *Proceedings of the National Academy of Sciences of the United States of America* 108 (7): 2975–80. https://doi.org/10.1073/pnas.1013031108.

Cutler, Horace G. 1988. “Trichothecenes and Their Role in the Expression of Plant Disease.” In , 50–72. https://doi.org/10.1021/bk-1988-0379.ch004.

Dagvadorj, Bayantes, Ahmet Caglar Ozketen, Ayse Andac, Cian Duggan, Tolga Osman Bozkurt, and Mahinur S. Akkaya. 2017. “A Puccinia Striiformis f. Sp. Tritici Secreted Protein Activates Plant Immunity at the Cell Surface.” *Scientific Reports* 7 (1): 1141. https://doi.org/10.1038/s41598-017-01100-z.

Danecek, Petr, Adam Auton, Goncalo Abecasis, Cornelis A Albers, Eric Banks, Mark A Depristo, Robert E Handsaker, et al. 2011. “The Variant Call Format and VCFtools.” *BIOINFORMATICS APPLICATIONS NOTE* 27 (15): 2156–58. https://doi.org/10.1093/bioinformatics/btr330.

Dardick, Chris, Benjamin Schwessinger, and Pamela Ronald. 2012. “Non-Arginine-Aspartate (Non-RD) Kinases Are Associated with Innate Immune Receptors That Recognize Conserved Microbial Signatures.” *Current Opinion in Plant Biology*. Elsevier Current Trends. https://doi.org/10.1016/j.pbi.2012.05.002.

Daudi, Arsalan, and Jose O’Brien. 2012. “Detection of Hydrogen Peroxide by DAB Staining in Arabidopsis Leaves.” *BIO-PROTOCOL* 2 (18). https://doi.org/10.21769/bioprotoc.263.

Daverdin, Guillaume, Thierry Rouxel, Lilian Gout, Jean-Noël Aubertot, Isabelle Fudal, Michel Meyer, Francis Parlange, Julien Carpezat, and Marie-Hélène Balesdent. 2012. “Genome Structure and Reproductive Behaviour Influence the Evolutionary Potential of a Fungal Phytopathogen.” Edited by Alex Andrianopoulos. *PLoS Pathogens* 8 (11): e1003020. https://doi.org/10.1371/journal.ppat.1003020.

Dawkins, Richard. 1999. *The Extended Phenotype: The Gene as the Unit of Selection*. Oxford: Oxford University Press (OUP). https://doi.org/10.1525/aa.1983.85.2.02a00540.

Dean, Ralph, Jan A L Van Kan, Zacharias A. Pretorius, Kim E. Hammond-Kosack, Antonio Di Pietro, Pietro D. Spanu, Jason J. Rudd, et al. 2012. “The Top 10 Fungal Pathogens in

Molecular Plant Pathology." *Molecular Plant Pathology* 13 (4): 414–30. https://doi.org/10.1111/j.1364-3703.2011.00783.x.

Deising, Holger B, Sven Reimann, and Sérgio F Pascholati. 2008. "Mechanisms and Significance of Fungicide Resistance." *Brazilian Journal of Microbiology* 39 (2): 286–95. https://doi.org/10.1590/s1517-838220080002000017.

Delourme, R., M. L. Pilet-Nayel, M. Archipiano, R. Horvais, X. Tanguy, T. Rouxel, H. Brun, M. Renard, and M. H. Balesdent. 2004. "A Cluster of Major Specific Resistance Genes to Leptosphaeria Maculans in Brassica Napus." *Phytopathology* 94 (6): 578–83. https://doi.org/10.1094/PHYTO.2004.94.6.578.

Dixon, Mark S., David A. Jones, James S. Keddie, Colwyn M. Thomas, Kate Harrison, and Jonathan D.G. Jones. 1996. "The Tomato Cf-2 Disease Resistance Locus Comprises Two Functional Genes Encoding Leucine-Rich Repeat Proteins." *Cell* 84 (3): 451–59. https://doi.org/10.1016/S0092-8674(00)81290-8.

Djamei, Armin, and Regine Kahmann. 2012. "Ustilago Maydis: Dissecting the Molecular Interface between Pathogen and Plant." Edited by Joseph Heitman. *PLoS Pathogens* 8 (11): e1002955. https://doi.org/10.1371/journal.ppat.1002955.

Djamei, Armin, Kerstin Schipper, Franziska Rabe, Anupama Ghosh, Volker Vincon, Jörg Kahnt, Sonia Osorio, Takayuki Tohge, Alisdair R Fernie, Ivo Feussner, Kirstin Feussner, Peter Meinicke, York-Dieter Stierhof, Heinz Schwarz, Boris Macek, et al. 2011. "Metabolic Priming by a Secreted Fungal Effector." *Nature* 1 (7369): 0–5. https://doi.org/10.1038/nature10454.

Djamei, Armin, Kerstin Schipper, Franziska Rabe, Anupama Ghosh, Volker Vincon, Jörg Kahnt, Sonia Osorio, Takayuki Tohge, Alisdair R. Fernie, Ivo Feussner, Kirstin Feussner, Peter Meinicke, York Dieter Stierhof, Heinz Schwarz, Boris MacEk, et al. 2011. "Metabolic Priming by a Secreted Fungal Effector." *Nature* 478 (7369): 395–98. https://doi.org/10.1038/nature10454.

Djulic, Alma, Annette Schmid, Heike Lenz, Pia Sharma, Christin Koch, Stefan G R Wirsel, Ralf T Voegele, and Daniel C Eastwood. 2011. "Transient Transformation of the Obligate Biotrophic Rust Fungus Uromyces Fabae Using Biolistics." *Fungal Biology* 115: 633–42. https://doi.org/10.1016/j.funbio.2011.03.007.

Doan, Thi Thai Yen, and Jeffrey Philip Obbard. 2012. "Enhanced Intracellular Lipid in Nannochloropsis Sp. via Random Mutagenesis and Flow Cytometric Cell Sorting." *Algal*

Research 1 (1): 17–21. https://doi.org/10.1016/j.algal.2012.03.001.

Dodds, P N, G J Lawrence, A M Catanzariti, M A Ayliffe, and J G Ellis. 2004. “The Melampsora Lini AvrL567 Avrirulence Genes Are Expressed n Hautoria and Their Products Are Recognized inside Plant Cells.” *The Plant Cell* 16 (April 2004): 755–68. https://doi.org/10.1105/tpc.020040.

Dodds, Peter N., and John P. Rathjen. 2010. “Plant Immunity: Towards an Integrated View of Plant–Pathogen Interactions.” *Nature Reviews Genetics* 11 (8): 539–48. https://doi.org/10.1038/nrg2812.

Doehlemann, Gunther, Karina Van Der Linde, Daniela Aßmann, Daniela Schwammbach, Alexander Hof, Amitabh Mohanty, David Jackson, and Regine Kahmann. 2009. “Pep1, a Secreted Effector Protein of Ustilago Maydis, Is Required for Successful Invasion of Plant Cells.” *PLoS Pathogens* 5 (2): e1000290. https://doi.org/10.1371/journal.ppat.1000290.

Dong, Suomeng, Sylvain Raffaele, and Sophien Kamoun. 2015. “The Two-Speed Genomes of Filamentous Pathogens: Waltz with Plants.” *Current Opinion in Genetics & Development* 35 (December): 57–65. https://doi.org/10.1016/j.gde.2015.09.001.

Dong, Suomeng, Weixiao Yin, Guanghui Kong, Xinyu Yang, Dinah Qutob, Qinghe Chen, Shiv D. Kale, et al. 2011. “Phytophthora Sojae Avirulence Effector Avr3b Is a Secreted NADH and ADP-Ribose Pyrophosphorylase That Modulates Plant Immunity.” Edited by David Mackey. *PLoS Pathogens* 7 (11): e1002353. https://doi.org/10.1371/journal.ppat.1002353.

Dracatos, Peter Michael, Jan Bartoȷ, Huda Elmansour, Davinder Singh, Miroslava Karafiátová, Peng Zhang, Burkhard Steuernagel, et al. 2019. “The Coiled-Coil NLR Rph1, Confers Leaf Rust Resistance in Barley Cultivar Sudan.” *Plant Physiology* 179 (4): 1362–72. https://doi.org/10.1104/PP.18.01052.

Dunker, Florian, Lorenz Oberkofler, Bernhard Lederer, Adriana Trutzenberg, and Arne Weiberg. 2021. “An Arabidopsis Downy Mildew Non-RxLR Effector Suppresses Induced Plant Cell Death to Promote Biotroph Infection.” Edited by Peter Bozhkov. *Journal of Experimental Botany* 72 (2): 718–32. https://doi.org/10.1093/jxb/eraa472.

Dunker, Florian, Adriana Trutzenberg, Jan Samuel Rothenpieler, Sarah Kuhn, Reinhard Pröls, Tom Schreiber, Alain Tissier, et al. 2020. “Oomycete Small RNAs Bind to the Plant RNA-Induced Silencing Complex for Virulence.” *ELife* 9. https://doi.org/10.7554/eLife.56096.

Duplessis, Sébastien, C. A. Cuomo, Y.-C. Lin, A. Aerts, E. Tisserant, C. Veneault-Fourrey, D. L. Joly, et al. 2011a. “Obligate Biotrophy Features Unraveled by the Genomic Analysis of Rust Fungi.” *Proceedings of the National Academy of Sciences* 108 (22): 9166–71. https://doi.org/10.1073/pnas.1019315108.

Duplessis, Sébastien, Christina A Cuomo, Y.-C. Lin, Andrea Aerts, Emilie Tisserant, Claire Veneault-Fourrey, David L Joly, et al. 2011b. “Obligate Biotrophy Features Unraveled by the Genomic Analysis of Rust Fungi.” *Proceedings of the National Academy of Sciences* 108 (22): 9166–71. https://doi.org/10.1073/pnas.1019315108.

Duxbury, Zane, Yan Ma, Oliver J. Furzer, Sung Un Huh, Volkan Cevik, Jonathan D.G. Jones, and Panagiotis F. Sarris. 2016. “Pathogen Perception by NLRs in Plants and Animals: Parallel Worlds.” *BioEssays*. John Wiley and Sons Inc. https://doi.org/10.1002/bies.201600046.

Duxbury, Zane, Shanshan Wang, Craig I. MacKenzie, Jeannette L. Tenthorey, Xiaoxiao Zhang, Sung Un Huh, Lanxi Hu, et al. 2020. “Induced Proximity of a TIR Signaling Domain on a Plant-Mammalian NLR Chimera Activates Defense in Plants.” *Proceedings of the National Academy of Sciences of the United States of America* 117 (31): 18832–39. https://doi.org/10.1073/pnas.2001185117.

Ellendorff, Ursula, Emilie F. Fradin, Ronnie De Jonge, and Bart P.H.J. Thomma. 2009. “RNA Silencing Is Required for Arabidopsis Defence against Verticillium Wilt Disease.” *Journal of Experimental Botany* 60 (2): 591–602. https://doi.org/10.1093/jxb/ern306.

Ellis, Jeffrey G., Maryam Rafiqi, Pamela Gan, Apratim Chakrabarti, and Peter N. Dodds. 2009. “Recent Progress in Discovery and Functional Analysis of Effector Proteins of Fungal and Oomycete Plant Pathogens.” *Current Opinion in Plant Biology* 12 (4): 399–405. https://doi.org/10.1016/j.pbi.2009.05.004.

Ellis, Jeffrey G, Evans S Lagudah, Wolfgang Spielmeyer, and Peter N Dodds. 2014. “The Past, Present and Future of Breeding Rust Resistant Wheat.” *Front Plant Sci* 5 (November): 641. https://doi.org/10.3389/fpls.2014.00641.

Emanuelsson, Olof, Henrik Nielsen, and Gunnar Von Heijne. 1999. “ChloroP, a Neural Network-Based Method for Predicting Chloroplast Transit Peptides and Their Cleavage Sites.” *Protein Science* 8 (5): 978–84. https://doi.org/10.1110/ps.8.5.978.

Esse, H P van, J W Van’t Klooster, M D Bolton, K A Yadeta, P van Baarlen, S Boeren, J Vervoort, P J de Wit, and B P Thomma. 2008. “The Cladosporium Fulvum Virulence

Protein Avr2 Inhibits Host Proteases Required for Basal Defense." *Plant Cell* 20 (7): 1948–63. https://doi.org/tpc.108.059394 [pii]\r10.1105/tpc.108.059394.

Eversmeyer, M. G., and C. L. Kramer. 2000. "Epidemiology of Wheat Leaf and Stem Rust in the Central Great Plains of the USA." *Annual Review of Phytopathology*. https://doi.org/10.1146/annurev.phyto.38.1.491.

FAO. 2019. "Crop Prospects and Food Situation - Quarterly Global Report No. 4." Rome. http://www.wipo.int/amc/en/mediation/rules.

Faraji, Javad. 2011. "Wheat Cultivar Blends: A Step Forward to Sustainable Agriculture." *African Journal of Agricultural Research* 6 (33): 6780–89. https://doi.org/10.5897/AJARX11.047.

Farfsing, Jan W., Kathrin Auffarth, and Christoph W. Basse. 2005a. "Identification of Cis-Active Elements in Ustilago Maydis Mig2 Promoters Conferring High-Level Activity during Pathogenic Growth in Maize." *Molecular Plant-Microbe Interactions* 18 (1): 75–87. https://doi.org/10.1094/MPMI-18-0075.

———. 2005b. "Identification of Cis-Active Elements in Ustilago Maydis Mig2 Promoters Conferring High-Level Activity during Pathogenic Growth in Maize." *Molecular Plant-Microbe Interactions* 18 (1): 75–87. https://doi.org/10.1094/MPMI-18-0075.

Faris, Justin D., Zengcui Zhang, Huangjun Lu, Shunwen Lu, Leela Reddy, Sylvie Cloutier, John P. Fellers, et al. 2010. "A Unique Wheat Disease Resistance-like Gene Governs Effector-Triggered Susceptibility to Necrotrophic Pathogens." *Proceedings of the National Academy of Sciences* 107 (30): 13544–49. https://doi.org/10.1073/PNAS.1004090107.

Farmers Guardian. 2019. "European Standing Committee Votes to Ban Fungicide Chlorothalonil - NEWS - Farmers Guardian." 2019. https://www.farminguk.com/news/european-commission-votes-to-ban-fungicide-chlorothalonil_51641.html.

Farrell, Andrew, Bradley I. Coleman, Brian Benenati, Kevin M. Brown, Ira J. Blader, Gabor T. Marth, and Marc Jan Gubbels. 2014. "Whole Genome Profiling of Spontaneous and Chemically Induced Mutations in Toxoplasma Gondii." *BMC Genomics* 15 (1): 354. https://doi.org/10.1186/1471-2164-15-354.

Feng, Hao, Ting Wang, Chuanxin Feng, Qiong Zhang, Xinmei Zhang, Lili Huang, Xiaojie Wang, and Zhensheng Kang. 2016. "Identification of MicroRNAs and Their Corresponding Targets Involved in the Susceptibility Interaction of Wheat Response to Puccinia

Striiformis f. Sp. Tritici." *Physiologia Plantarum* 157 (1): 95–107. https://doi.org/10.1111/ppl.12407.

Feng, Jie, Guosheng Liu, Gopalan Selvaraj, Geoffrey R. Hughes, and Yangdou Wei. 2005. "A Secreted Lipase Encoded by LIP1 Is Necessary for Efficient Use of Saturated Triglyceride Lipids in Fusarium Graminearum." *Microbiology* 151 (12): 3911–21. https://doi.org/10.1099/mic.0.28261-0.

Fernandez, Diana, Emilie Tisserant, Pedro Talhinhas, Helena Azinheira, Ana Vieira, Anne Sophie Petitot, Andreia Loureiro, et al. 2012. "454-Pyrosequencing of Coffea Arabica Leaves Infected by the Rust Fungus Hemileia Vastatrix Reveals in Planta-Expressed Pathogen-Secreted Proteins and Plant Functions in a Late Compatible Plant-Rust Interaction." *Molecular Plant Pathology* 13 (1): 17–37. https://doi.org/10.1111/j.1364-3703.2011.00723.x.

Feuillet, Catherine, Silvia Travella, Nils Stein, Laurence Albar, Aurélie Nublat, and Beat Keller. 2003. "Map-Based Isolation of the Leaf Rust Disease Resistance Gene Lr10 from the Hexaploid Wheat (Triticum Aestivum L.) Genome." *Proceedings of the National Academy of Sciences of the United States of America* 100 (25): 15253–58. https://doi.org/10.1073/pnas.2435133100.

Ficarra, Florencia A., Carolina Grandellis, Betiana S. Garavaglia, Natalia Gottig, and Jorgelina Ottado. 2018. "Bacterial and Plant Natriuretic Peptides Improve Plant Defence Responses against Pathogens." *Molecular Plant Pathology* 19 (4): 801–11. https://doi.org/10.1111/mpp.12560.

Figueroa, Melania, Peter N. Dodds, and Eva C. Henningsen. 2020a. "Evolution of Virulence in Rust Fungi — Multiple Solutions to One Problem." *Current Opinion in Plant Biology*. Elsevier Ltd. https://doi.org/10.1016/j.pbi.2020.02.007.

———. 2020b. "Evolution of Virulence in Rust Fungi — Multiple Solutions to One Problem." *Current Opinion in Plant Biology* 56 (August): 20–27. https://doi.org/10.1016/j.pbi.2020.02.007.

Figueroa, Melania, Kim E. Hammond-Kosack, and Peter S. Solomon. 2018. "A Review of Wheat Diseases—a Field Perspective." *Molecular Plant Pathology*. Blackwell Publishing Ltd. https://doi.org/10.1111/mpp.12618.

Fischer, Iris, Anne Diévart, Gaetan Droc, Jean François Dufayard, and Nathalie Chantret. 2016. "Evolutionary Dynamics of the Leucine-Rich Repeat Receptor-like Kinase (LRR-

RLK) Subfamily in Angiosperms." *Plant Physiology* 170 (3): 1595–1610. https://doi.org/10.1104/pp.15.01470.

Flor, HH. 1942. "Inheritance of Pathogenicity in Melampsora Lini." *Phytopathology* 32: 653–69. https://ci.nii.ac.jp/naid/10024791669.

———. 1955. "Host-Parasite Interactions in Flax Rust: Its Genetics and Other Implications." *Phytopathology* 45: 680–85.

Ford-Lloyd, Brian V., Markus Schmidt, Susan J. Armstrong, Oz Barazani, Jan Engels, Rivka Hadas, Karl Hammer, et al. 2011. "Crop Wild Relatives—Undervalued, Underutilized and under Threat?" *BioScience* 61 (7): 559–65. https://doi.org/10.1525/bio.2011.61.7.10.

Forsberg, Åke, Ingrid Bölin, Lena Norlander, and Hans Wolf-Watz. 1987. "Molecular Cloning and Expression of Calcium-Regulated, Plasmid-Coded Proteins of Y. Pseudotuberculosis." *Microbial Pathogenesis* 2 (2): 123–37. https://doi.org/10.1016/0882-4010(87)90104-5.

Fouché, Simone, Clémence Plissonneau, and Daniel Croll. 2018. "The Birth and Death of Effectors in Rapidly Evolving Filamentous Pathogen Genomes." *Current Opinion in Microbiology*. Elsevier Ltd. https://doi.org/10.1016/j.mib.2018.01.020.

Franceschetti, Marina, Abbas Maqbool, Maximiliano J Jiménez-Dalmaroni, Helen G Pennington, Sophien Kamoun, and Mark J Banfield. 2017. "Effectors of Filamentous Plant Pathogens: Commonalities amid Diversity." *Microbiology and Molecular Biology Reviews* 81 (2): e00066-16. https://doi.org/10.1128/mmbr.00066-16.

Friesen, T. L., D. J. Holmes, R. L. Bowden, and J. D. Faris. 2018. "ToxA Is Present in the U.S. Bipolaris Sorokiniana Population and Is a Significant Virulence Factor on Wheat Harboring Tsn1." *Plant Disease* 102 (12): 2446–52. https://doi.org/10.1094/pdis-03-18-0521-re.

Friesen, Timothy L., Eva H. Stukenbrock, Zhaohui Liu, Steven Meinhardt, Hua Ling, Justin D. Faris, Jack B. Rasmussen, Peter S. Solomon, Bruce A. McDonald, and Richard P. Oliver. 2006. "Emergence of a New Disease as a Result of Interspecific Virulence Gene Transfer." *Nature Genetics* 38 (8): 953–56. https://doi.org/10.1038/ng1839.

Fu, Daolin, Cristobal Uauy, Assaf Distelfeld, Ann Blechl, Lynn Epstein, Xianming Chen, Hanan Sela, Tzion Fahima, and Jorge Dubcovsky. 2009. "A Kinase-START Gene Confers Temperature-Dependent Resistance to Wheat Stripe Rust." *Science* 323 (5919): 1357–

60. https://doi.org/10.1126/science.1166289.

Fudal, I., S. Ross, L. Gout, F. Blaise, M. L. Kuhn, M. R. Eckert, L. Cattolico, S. Bernard-Samain, M. H. Balesdent, and Thierry Rouxel. 2007. “Heterochromatin-like Regions as Ecological Niches for Avirulence Genes in the Leptosphaeria Maculans Genome: Map-Based Cloning of AvrLm6.” *Molecular Plant-Microbe Interactions* 20 (4): 459–70. https://doi.org/10.1094/MPMI-20-4-0459.

Fukuoka, Shuichi, Norikuni Saka, Yuko Mizukami, Hironori Koga, Utako Yamanouchi, Yosuke Yoshioka, Nagao Hayashi, Kaworu Ebana, Ritsuko Mizobuchi, and Masahiro Yano. 2015. “Gene Pyramiding Enhances Durable Blast Disease Resistance in Rice.” *Scientific Reports* 5 (January): 7773. https://doi.org/10.1038/srep07773.

Gan, Pamela, Kyoko Ikeda, Hiroki Irieda, Mari Narusaka, Richard J. O’Connell, Yoshihiro Narusaka, Yoshitaka Takano, Yasuyuki Kubo, and Ken Shirasu. 2013. “Comparative Genomic and Transcriptomic Analyses Reveal the Hemibiotrophic Stage Shift of Colletotrichum Fungi.” *New Phytologist* 197 (4): 1236–49. https://doi.org/10.1111/nph.12085.

Garnica, Diana P., Adnane Nemri, Narayana M. Upadhyaya, John P. Rathjen, and Peter N. Dodds. 2014. “The Ins and Outs of Rust Haustoria.” Edited by Joseph Heitman. *PLoS Pathogens* 10 (9): e1004329. https://doi.org/10.1371/journal.ppat.1004329.

GBIF. 2017. “GBIF Backbone Taxonomy.” GBIF. 2017. https://doi.org/https://doi.org/10.5072/hufs9m.

Ge, Xiaochun, Guo Jing Li, Sheng Bing Wang, Huifen Zhu, Tong Zhu, Xun Wang, and Yiji Xia. 2007. “AtNUDT7, a Negative Regulator of Basal Immunity in Arabidopsis, Modulates Two Distinct Defense Response Pathways and Is Involved in Maintaining Redox Homeostasis.” *Plant Physiology* 145 (1): 204–15. https://doi.org/10.1104/pp.107.103374.

Gepts, Paul, Sambasivam Periyannan, Caiguo Zhang, Lesley A. Boyd, van H Schalkwyk, Renée Prins, Susanne Dreisigacker, et al. 2016. “Stem Rust Resistance in a Geographically Diverse Collection of Spring Wheat Lines Collected from Stem Rust Resistance in a Geographically Diverse Collection of Spring Wheat Lines Collected from Across Africa.” *Frontiers in Plant Science | Www.Frontiersin.Org 1 Front. Plant Sci* 7 (7): 973. https://doi.org/10.3389/fpls.2016.00973.

Giraldo, Martha C, Yasin F Dagdas, Yogesh K Gupta, Thomas a Mentlak, Mihwa Yi, Ana Lilia

Martinez-Rocha, Hiromasa Saitoh, Ryohei Terauchi, Nicholas J Talbot, and Barbara Valent. 2013. “Two Distinct Secretion Systems Facilitate Tissue Invasion by the Rice Blast Fungus Magnaporthe Oryzae.” *Nature Communications* 4 (May): 1996. https://doi.org/10.1038/ncomms2996.

Godfray, H Charles J, John R Beddington, Ian R Crute, Lawrence Haddad, David Lawrence, James F Muir, Jules Pretty, Sherman Robinson, Sandy M Thomas, and Camilla Toulmin. 2010. “Food Security: The Challenge of Feeding 9 Billion People.” *Science* 327 (5967): 812–18. https://doi.org/10.1126/science.1185383.

Godfrey, Dale, Henrik Böhlenius, Carsten Pedersen, Ziguo Zhang, Jeppe Emmersen, and Hans Thordal-Christensen. 2010. “Powdery Mildew Fungal Effector Candidates Share N-Terminal Y/F/WxC-Motif.” *BMC Genomics* 11 (1): 317. https://doi.org/10.1186/1471-2164-11-317.

González-Fernández, Raquel, Elena Prats, and Jesús V. Jorrín-Novo. 2010. “Proteomics of Plant Pathogenic Fungi.” *Journal of Biomedicine & Biotechnology* 2010: 932527. https://doi.org/10.1155/2010/932527.

Gout, Lilian, Isabelle Fudal, Marie Line Kuhn, Françoise Blaise, Maria Eckert, Laurence Cattolico, Marie Hélène Balesdent, and Thierry Rouxel. 2006. “Lost in the Middle of Nowhere: The AvrLm1 Avirulence Gene of the Dothideomycete Leptosphaeria Maculans.” *Molecular Microbiology* 60 (1): 67–80. https://doi.org/10.1111/j.1365-2958.2006.05076.x.

Gout, Lilian, Marie Line Kuhn, Lucie Vincenot, Sylvie Bernard-Samain, Laurence Cattolico, Martin Barbetti, Onésimo Moreno-Rico, Marie Hélène Balesdent, and Thierry Rouxel. 2007. “Genome Structure Impacts Molecular Evolution at the AvrLm1 Avirulence Locus of the Plant Pathogen Leptosphaeria Maculans.” *Environmental Microbiology* 9 (12): 2978–92. https://doi.org/10.1111/j.1462-2920.2007.01408.x.

Green, G. J. 1981. “Identification of Physiologic Races of Puccinia Graminis f. Sp. Tritici in Canada.” *Canadian Journal of Plant Pathology*. Vol. 3. https://doi.org/10.1080/07060668109501400.

Greenberg, Jean T. 1997. “PROGRAMMED CELL DEATH IN PLANT-PATHOGEN INTERACTIONS.” *Annual Review of Plant Physiology and Plant Molecular Biology* 48 (June): 525–45. https://doi.org/10.1146/annurev.arplant.48.1.525.

Gu, Biao, Shiv D. Kale, Qinhu Wang, Dinghe Wang, Qiaona Pan, Hua Cao, Yuling Meng,

Zhensheng Kang, Brett M. Tyler, and Weixing Shan. 2011. “Rust Secreted Protein Ps87 Is Conserved in Diverse Fungal Pathogens and Contains a RXLR-like Motif Sufficient for Translocation into Plant Cells.” Edited by Kirsten Nielsen. *PLoS ONE* 6 (11): e27217. https://doi.org/10.1371/journal.pone.0027217.

Guardian, Farmers. 2020. “Rare Cereal Disease Found in Trial Plots at Oak Park - NEWS - Farmers Guardian.” Farmers Guardian. 2020. https://www.fginsight.com/news/rare-cereal-disease-found-in-trial-plots-at-oak-park-110788.

Guillen, Karine de, Diana Ortiz-Vallejo, Jérome Gracy, Elisabeth Fournier, Thomas Kroj, André Padilla, and J Heitman. 2015. “Structure Analysis Uncovers a Highly Diverse but Structurally Conserved Effector Family in Phytopathogenic Fungi.” Edited by Jin-Rong Xu. *PLOS Pathogens* 11 (10): e1005228. https://doi.org/10.1371/journal.ppat.1005228.

Guo, Ya Long, Joffrey Fitz, Korbinian Schneeberger, Stephan Ossowski, Jun Cao, and Detlef Weigel. 2011. “Genome-Wide Comparison of Nucleotide-Binding Site-Leucine-Rich Repeat-Encoding Genes in Arabidopsis.” *Plant Physiology* 157 (2): 757–69. https://doi.org/10.1104/pp.111.181990.

Guttman, David S., Alice C. McHardy, and Paul Schulze-Lefert. 2014. “Microbial Genome-Enabled Insights into Plant-Microorganism Interactions.” *Nature Reviews Genetics*. Nature Publishing Group. https://doi.org/10.1038/nrg3748.

Haas, Brian J., Sophien Kamoun, Michael C. Zody, Rays H.Y. Jiang, Robert E. Handsaker, Liliana M. Cano, Manfred Grabherr, et al. 2009. “Genome Sequence and Analysis of the Irish Potato Famine Pathogen Phytophthora Infestans.” *Nature* 461 (7262): 393–98. https://doi.org/10.1038/nature08358.

Hafiz Khan, Mudasir, Asifa Bukhari, Ahmad Dar, and Syed Mudasir Rizvi. 2013. “Status and Strategies in Breeding for Rust Resistance in Wheat” 4 (6): 292–301. https://doi.org/10.4236/as.2013.46042.

Hann, Dagmar R., and John P. Rathjen. 2007. “Early Events in the Pathogenicity of Pseudomonas Syringae on Nicotiana Benthamiana.” *Plant Journal* 49 (4): 607–18. https://doi.org/10.1111/j.1365-313X.2006.02981.x.

Harris, C. Jake, Erik J. Slootweg, Aska Goverse, and David C. Baulcombe. 2013. “Stepwise Artificial Evolution of a Plant Disease Resistance Gene.” *Proceedings of the National Academy of Sciences of the United States of America* 110 (52): 21189–94. https://doi.org/10.1073/pnas.1311134110.

Harvell, C D. 2002. “Climate Warming and Disease Risks for Terrestrial and Marine Biota.” *Science Magazine* 296 (5576).

Hauck, Paula, Roger Thilmony, and Sheng Yang He. 2003. “A Pseudomonas Syringae Type III Effector Suppresses Cell Wall-Based Extracellular Defense in Susceptible Arabidopsis Plants.” *Proceedings of the National Academy of Sciences of the United States of America* 100 (14): 8577–82. https://doi.org/10.1073/pnas.1431173100.

Haudry, A., A. Cenci, C. Ravel, T. Bataillon, D. Brunel, C. Poncet, I. Hochu, et al. 2007. “Grinding up Wheat: A Massive Loss of Nucleotide Diversity since Domestication.” *Molecular Biology and Evolution* 24 (7): 1506–17. https://doi.org/10.1093/molbev/msm077.

He, Sheng Yang, Kinya Nomura, and Thomas S. Whittam. 2004. “Type III Protein Secretion Mechanism in Mammalian and Plant Pathogens.” *Biochimica et Biophysica Acta - Molecular Cell Research*. Elsevier. https://doi.org/10.1016/j.bbamcr.2004.03.011.

Heck, Carolin, Hannah Kuhn, Sven Heidt, Stefanie Walter, Nina Rieger, and Natalia Requena. 2016. “Symbiotic Fungi Control Plant Root Cortex Development through the Novel GRAS Transcription Factor MIG1.” *Current Biology* 26 (20): 2770–78. https://doi.org/10.1016/j.cub.2016.07.059.

Hemetsberger, Christoph, Christian Herrberger, Bernd Zechmann, Morten Hillmer, and Gunther Doehlemann. 2012a. “The Ustilago Maydis Effector Pep1 Suppresses Plant Immunity by Inhibition of Host Peroxidase Activity.” *PLoS Pathogens* 8 (5): e1002684. https://doi.org/10.1371/journal.ppat.1002684.

———. 2012b. “The Ustilago Maydis Effector Pep1 Suppresses Plant Immunity by Inhibition of Host Peroxidase Activity.” Edited by Jin-Rong Xu. *PLoS Pathogens* 8 (5): e1002684. https://doi.org/10.1371/journal.ppat.1002684.

Hester, Susan D., Douglas C. Wolf, Stephen Nesnow, and Sheau Fung Thai. 2006. “Transcriptional Profiles in Liver from Rats Treated with Tumorigenic and Non-Tumorigenic Triazole Conazole Fungicides: Propiconazole, Triadimefon, and Myclobutanil.” *Toxicologic Pathology* 34 (7): 879–94. https://doi.org/10.1080/01926230601047824.

Hester, Susan, Tanya Moore, William T. Padgett, Lynea Murphy, Charles E. Wood, and Stephen Nesnow. 2012. “The Hepatocarcinogenic Conazoles: Cyproconazole, Epoxiconazole, and Propiconazole Induce a Common Set of Toxicological and

Transcriptional Responses." *Toxicological Sciences* 127 (1): 54–65. https://doi.org/10.1093/toxsci/kfs086.

Hickey, Lee T., Amber N. Hafeez, Hannah Robinson, Scott A. Jackson, Soraya C.M. Leal-Bertioli, Mark Tester, Caixia Gao, Ian D. Godwin, Ben J. Hayes, and Brande B.H. Wulff. 2019. "Breeding Crops to Feed 10 Billion." *Nature Biotechnology*. Nature Research. https://doi.org/10.1038/s41587-019-0152-9.

Hiebert, Colin W., James A. Kolmer, Curt A. McCartney, Jordan Briggs, Tom Fetch, Harbans Bariana, Frederic Choulet, Matthew N. Rouse, and Wolfgang Spielmeyer Spielmeyer. 2016. "Major Gene for Field Stem Rust Resistance Co-Locates with Resistance Gene Sr12 in 'thatcher' Wheat." *PLoS ONE* 11 (6). https://doi.org/10.1371/journal.pone.0157029.

Hogenhout, Saskia A., and Jorunn I B Bos. 2011. "Effector Proteins That Modulate Plant-Insect Interactions." *Current Opinion in Plant Biology* 14 (4): 422–28. https://doi.org/10.1016/j.pbi.2011.05.003.

Hogenhout, Saskia a, Renier a L Van der Hoorn, Ryohei Terauchi, and Sophien Kamoun. 2009. "Emerging Concepts in Effector Biology of Plant-Associated Organisms." *Molecular Plant-Microbe Interactions : MPMI* 22 (2): 115–22. https://doi.org/10.1094/MPMI-22-2-0115.

Hoorn, Renier A.L. Van Der, and Sophien Kamoun. 2008. "From Guard to Decoy: A New Model for Perception of Plant Pathogen Effectors." *Plant Cell* 20 (8): 2009–17. https://doi.org/10.1105/tpc.108.060194.

Hörger, Anja C., Muhammad Ilyas, Wolfgang Stephan, Aurélien Tellier, Renier A. L. van der Hoorn, and Laura E. Rose. 2012. "Balancing Selection at the Tomato RCR3 Guardee Gene Family Maintains Variation in Strength of Pathogen Defense." Edited by Rodney Mauricio. *PLoS Genetics* 8 (7): e1002813. https://doi.org/10.1371/journal.pgen.1002813.

Horton, Paul, Keun Joon Park, Takeshi Obayashi, Naoya Fujita, Hajime Harada, C. J. Adams-Collier, and Kenta Nakai. 2007. "WoLF PSORT: Protein Localization Predictor." *Nucleic Acids Research* 35 (SUPPL.2). https://doi.org/10.1093/nar/gkm259.

Hou, Yingnan, Yi Zhai, Li Feng, Hana Z Karimi, Brian D Rutter, Liping Zeng, Du Seok Choi, et al. 2019. "A Phytophthora Effector Suppresses Trans-Kingdom RNAi to Promote Disease Susceptibility." https://doi.org/10.1016/j.chom.2018.11.007.

Houterman, Petra M., Ben J.C. Cornelissen, and Martijn Rep. 2008. “Suppression of Plant Resistance Gene-Based Immunity by a Fungal Effector.” *PLoS Pathogens* 4 (5): 1000061. https://doi.org/10.1371/journal.ppat.1000061.

Hovmøller, M. S., A. F. Justesen, and J. K M Brown. 2002. “Clonality and Long-Distance Migration of Puccinia Striiformis f.Sp. Tritici in North-West Europe.” *Plant Pathology* 51 (1): 24–32. https://doi.org/10.1046/j.1365-3059.2002.00652.x.

Hovmøller, Mogens S., and Annemarie F. Justesen. 2007. “Appearance of Atypical Puccinia Striiformis f. Sp. Tritici Phenotypes in North-Western Europe.” *Australian Journal of Agricultural Research* 58 (6): 518. https://doi.org/10.1071/AR06146.

Huang, Ju, Weina Si, Qiming Deng, Ping Li, and Sihai Yang. 2014. “Rapid Evolution of Avirulence Genes in Rice Blast Fungus Magnaporthe Oryzae.” *BMC Genetics* 15 (1): 45. https://doi.org/10.1186/1471-2156-15-45.

Huang, Li, Steven A. Brooks, Wanlong Li, John P. Fellers, Harold N. Trick, and Bikram S. Gill. 2003. “Map-Based Cloning of Leaf Rust Resistance Gene Lr21 from the Large and Polyploid Genome of Bread Wheat.” *Genetics* 164 (2): 655–64. http://www.cimmyt.org/.

Huang, Y. J., Z. Q. Li, N Evans, T Rouxel, B. D L Fitt, and M. H. Balesdent. 2006. “Fitness Cost Associated with Loss of the AvrLm4 Avirulence Function in Leptosphaeria Maculans (Phoma Stem Canker of Oilseed Rape).” In *European Journal of Plant Pathology*, 114:77–89. https://doi.org/10.1007/s10658-005-2643-4.

Huckelhoven, Ralph, Cornelia Dechert, and Karl-Heinz Kogel. 2001. “Non-Host Resistance of Barley Is Associated with a Hydrogen Peroxide Burst at Sites of Attempted Penetration by Wheat Powdery Mildew Fungus.” *Molecular Plant Pathology* 2 (4): 199–205. https://doi.org/10.1046/j.1464-6722.2001.00067.x.

Huerta-Espino, J., R. P. Singh, S. Germán, B. D. McCallum, R. F. Park, W. Q. Chen, S. C. Bhardwaj, and H. Goyeau. 2011. “Global Status of Wheat Leaf Rust Caused by Puccinia Triticina.” *Euphytica* 179 (1): 143–60. https://doi.org/10.1007/s10681-011-0361-x.

Hurni, Severine, Susanne Brunner, Daniel Stirnweis, Gerhard Herren, David Peditto, Robert A. McIntosh, and Beat Keller. 2014. “The Powdery Mildew Resistance Gene *Pm8* Derived from Rye Is Suppressed by Its Wheat Ortholog *Pm3*.” *The Plant Journal* 79 (6): 904–13. https://doi.org/10.1111/tpj.12593.

Ikehata, Hironobu, and Tetsuya Ono. 2011. “The Mechanisms of UV Mutagenesis.” *Journal*

of Radiation Research 52 (2): 115–25. https://doi.org/10.1269/jrr.10175.

Islam, M. Tofazzal, Daniel Croll, Pierre Gladieux, Darren M. Soanes, Antoine Persoons, Pallab Bhattacharjee, Md. Shaid Hossain, et al. 2016. "Emergence of Wheat Blast in Bangladesh Was Caused by a South American Lineage of Magnaporthe Oryzae." *BMC Biology* 14 (1): 84. https://doi.org/10.1186/s12915-016-0309-7.

Jacob, Florence, Saskia Vernaldi, and Takaki Maekawa. 2013. "Evolution and Conservation of Plant NLR Functions." *Frontiers in Immunology*. Frontiers Media SA. https://doi.org/10.3389/fimmu.2013.00297.

Jacob, Pierre, Hyun Kim, Feihua Wu, Farid El-Kasmi, William G. Walton, Oliver J. Furzer, Adam D. Lietzen, et al. 2021. "The Plant Immune Receptors NRG1.1 and ADR1 Are Calcium Influx Channels." *BioRxiv*, February, 2021.02.25.431980. https://doi.org/10.1101/2021.02.25.431980.

Jia, Yulin, Sean A McAdams, Gregory T Bryan, Howard P Hershey, and Barbara Valent. 2000. "Direct Interaction of Resistance Gene and Avirulence Gene Products Confers Rice Blast Resistance." *EMBO Journal* 19 (15): 4004–14. https://doi.org/10.1093/emboj/19.15.4004.

Jiang, Jiming, Bernd Friebe, and Bikram S. Gill. 1993. "Recent Advances in Alien Gene Transfer in Wheat." *Euphytica* 73 (3): 199–212. https://doi.org/10.1007/BF00036700.

Jiang, Rays H.Y., Sucheta Tripathy, Francine Govers, and Brett M. Tyler. 2008a. "RXLR Effector Reservoir in Two Phytophthora Species Is Dominated by a Single Rapidly Evolving Superfamily with More than 700 Members." *Proceedings of the National Academy of Sciences of the United States of America* 105 (12): 4874–79. https://doi.org/10.1073/pnas.0709303105.

———. 2008b. "RXLR Effector Reservoir in Two Phytophthora Species Is Dominated by a Single Rapidly Evolving Superfamily with More than 700 Members." *Proceedings of the National Academy of Sciences of the United States of America* 105 (12): 4874–79. https://doi.org/10.1073/pnas.0709303105.

Jin, Y., R. P. Singh, R. W. Ward, R. Wanyera, M. Kinyua, P. Njau, T. Fetch, Z. A. Pretorius, and A. Yahyaoui. 2007. "Characterization of Seedling Infection Types and Adult Plant Infection Responses of Monogenic Sr Gene Lines to Race TTKS of Puccinia Graminis f. Sp. Tritici." *Plant Disease* 91 (9): 1096–99. https://doi.org/10.1094/PDIS-91-9-1096.

Jin, Y., L. J. Szabo, Z. A. Pretorius, R. P. Singh, R. Ward, and T. Fetch. 2008a. "Detection of

Virulence to Resistance Gene Sr24 Within Race TTKS of Puccinia Graminis f. Sp. Tritici." *Plant Disease* 92 (6): 923–26. https://doi.org/10.1094/PDIS-92-6-0923.

———. 2008b. "Detection of Virulence to Resistance Gene *Sr24* Within Race TTKS of *Puccinia Graminis* f. Sp. *Tritici*." *Plant Disease* 92 (6): 923–26. https://doi.org/10.1094/PDIS-92-6-0923.

Johnson, T. 1954. "SELFING STUDIES WITH PHYSIOLOGIC RACES OF WHEAT STEM RUST, PUCCINIA GRAMINIS VAR TRITICI." *Canadian Journal of Botany* 32 (4): 506–22. https://doi.org/10.1139/b54-047.

———. 1961. "Man-Guided Evolution in Plant Rusts: Through His Modification of the Host Plants of the Cereal Rusts, Man Is Also Modifying the Rusts." *Science* 133 (3450): 357–62. https://doi.org/10.1126/science.133.3450.357.

Jones, D A. 1988. "Genetic Properties of Inhibitor Genes in Flax Rust That Alter Avirulence to Virulence on Flax." *Phytopathology* 78 (3): 342. https://doi.org/10.1094/phyto-78-342.

Jones, David A., and Daigo Takemoto. 2004. "Plant Innate Immunity - Direct and Indirect Recognition of General and Specific Pathogen-Associated Molecules." *Current Opinion in Immunology*. https://doi.org/10.1016/j.coi.2003.11.016.

Jones, J. D. G., R. E. Vance, and J. L. Dangl. 2016. "Intracellular Innate Immune Surveillance Devices in Plants and Animals." *Science* 354 (6316): aaf6395–aaf6395. https://doi.org/10.1126/science.aaf6395.

Jones, Jonathan D.G., and Jeffery L. Dangl. 2006. "The Plant Immune System." *Nature*. https://doi.org/10.1038/nature05286.

Jones, Jonathan D G, and Jeffery L Dangl. 2006. "The Plant Immune System." *Nature* 444 (7117): 323–29. https://doi.org/10.1038/nature05286.

Jonge, Ronnie De, H. Peter Van Esse, Karunakaran Maruthachalam, Melvin D. Bolton, Parthasarathy Santhanam, Mojtaba Keykha Saber, Zhao Zhang, et al. 2012. "Tomato Immune Receptor Ve1 Recognizes Effector of Multiple Fungal Pathogens Uncovered by Genome and RNA Sequencing." *Proceedings of the National Academy of Sciences of the United States of America* 109 (13): 5110–15. https://doi.org/10.1073/pnas.1119623109.

Juroszek, Peter, and Andreas Tiedemann. 2013. "Climate Change and Potential Future Risks through Wheat Diseases: A Review." *European Journal of Plant Pathology* 136 (1): 21–33. https://doi.org/10.1007/s10658-012-0144-9.

Kadota, Yasuhiro, Ken Shirasu, and Cyril Zipfel. 2015. “Regulation of the NADPH Oxidase RBOHD during Plant Immunity.” *Plant and Cell Physiology* 56 (8): 1472–80. https://doi.org/10.1093/pcp/pcv063.

Kale, Shiv D., Biao Gu, Daniel G.S. Capelluto, Daolong Dou, Emily Feldman, Amanda Rumore, Felipe D. Arredondo, et al. 2010. “External Lipid PI3P Mediates Entry of Eukaryotic Pathogen Effectors into Plant and Animal Host Cells.” *Cell* 142 (2): 284–95. https://doi.org/10.1016/j.cell.2010.06.008.

Kamoun, Sophien. 2006. “A Catalogue of the Effector Secretome of Plant Pathogenic Oomycetes.” https://doi.org/10.1146/annurev.phyto.44.070505.143436.

Kangara, Ngonidzashe, Tomasz J. Kurowski, Guru V. Radhakrishnan, Sreya Ghosh, Nicola M. Cook, Guotai Yu, Sanu Arora, et al. 2020. “Mutagenesis of Puccinia Graminis f. Sp. Tritici and Selection of Gain-of-Virulence Mutants.” *Frontiers in Plant Science* 11 (September): 1. https://doi.org/10.3389/fpls.2020.570180.

Kanyuka, Kostya, and Jason J. Rudd. 2019. “Cell Surface Immune Receptors: The Guardians of the Plant’s Extracellular Spaces.” *Current Opinion in Plant Biology* 50 (August): 1–8. https://doi.org/10.1016/j.pbi.2019.02.005.

Kawamura, Kazuto, and Ichiro N. Maruyama. 2019. “Forward Genetic Screen for Caenorhabditis Elegans Mutants with a Shortened Locomotor Healthspan.” *G3: Genes, Genomes, Genetics* 9 (8): 2415–23. https://doi.org/10.1534/g3.119.400241.

Kawashima, Cintia G, Gustavo Augusto Guimarães, Sônia Regina Nogueira, Dan MacLean, Doug R Cook, Burkhard Steuernagel, Jongmin Baek, et al. 2016. “A Pigeonpea Gene Confers Resistance to Asian Soybean Rust in Soybean.” *Nature Biotechnology* 34 (6): 661–65. https://doi.org/10.1038/nbt.3554.

Kazan, Kemal, and Rebecca Lyons. 2014. “Intervention of Phytohormone Pathways by Pathogen Effectors.” *Plant Cell*. American Society of Plant Biologists. https://doi.org/10.1105/tpc.114.125419.

Keller, Beat, Thomas Wicker, and Simon G. Krattinger. 2018. “Advances in Wheat and Pathogen Genomics: Implications for Disease Control.” *Annual Review of Phytopathology*. Annual Reviews. https://doi.org/10.1146/annurev-phyto-080516-035419.

Kemen, Eric, Ariane C. Kemen, Maryam Rafiqi, Uta Hempel, Kurt Mendgen, Matthias Hahn, and Ralf T. Voegele. 2005. “Identification of a Protein from Rust Fungi Transferred from

Haustoria into Infected Plant Cells." *Molecular Plant-Microbe Interactions* 18 (11): 1130–39. https://doi.org/10.1094/MPMI-18-1130.

Kemen, Eric, Ariane Kemen, Andreas Ehlers, Ralf Voegele, and Kurt Mendgen. 2013. "A Novel Structural Effector from Rust Fungi Is Capable of Fibril Formation." *The Plant Journal* 75 (5): 767–80. https://doi.org/10.1111/tpj.12237.

Keshavarzi, Mansureh, Soner Soylu, Ian Brown, Ulla Bonas, Michel Nicole, John Rossiter, and John Mansfield. 2004. "Basal Defense Induced in Pepper by Lipopolysaccharides Are Suppressed by Xanthomonas Campestris Pv. Vesicatoria." *Molecular Plant-Microbe Interactions* 17 (7): 805–15. https://doi.org/10.1094/MPMI.2004.17.7.805.

Kim, Min Gab, Luis Da Cunha, Aidan J. McFall, Youssef Belkhadir, Sruti DebRoy, Jeffrey L. Dangl, and David Mackey. 2005. "Two Pseudomonas Syringae Type III Effectors Inhibit RIN4-Regulated Basal Defense in Arabidopsis." *Cell* 121 (5): 749–59. https://doi.org/10.1016/j.cell.2005.03.025.

Kim, Sang Hee, Dong Qi, Tom Ashfield, Matthew Helm, and Roger W. Innes. 2016. "Using Decoys to Expand the Recognition Specificity of a Plant Disease Resistance Protein." *Science* 351 (6274): 684–87. https://doi.org/10.1126/science.aad3436.

Kinsey, J A, P W Garrett-Engele, E B Cambareri, and E U Selker. 1994. "The Neurospora Transposon Tad Is Sensitive to Repeat-Induced Point Mutation (RIP)." *Genetics* 138 (3).

Kleemann, Jochen, Linda J. Rincon-Rivera, Hiroyuki Takahara, Ulla Neumann, Emiel Ver Loren van Themaat, H. Charlotte van der Does, Stéphane Hacquard, et al. 2012. "Sequential Delivery of Host-Induced Virulence Effectors by Appressoria and Intracellular Hyphae of the Phytopathogen Colletotrichum Higginsianum." *PLoS Pathogens* 8 (4). https://doi.org/10.1371/journal.ppat.1002643.

Klessig, Daniel F., Hyong Woo Choi, and D'Maris Amick Dempsey. 2018. "Systemic Acquired Resistance and Salicylic Acid: Past, Present, and Future." *Molecular Plant-Microbe Interactions®* 31 (9): 871–88. https://doi.org/10.1094/MPMI-03-18-0067-CR.

Klymiuk, Valentina, Elitsur Yaniv, Lin Huang, Dina Raats, Andrii Fatiukha, Shisheng Chen, Lihua Feng, et al. 2018. "Cloning of the Wheat Yr15 Resistance Gene Sheds Light on the Plant Tandem Kinase-Pseudokinase Family." *Nature Communications* 9 (1): 3735. https://doi.org/10.1038/s41467-018-06138-9.

Koeck, Markus, Adrienne R. Hardham, and Peter N. Dodds. 2011. "The Role of Effectors of Biotrophic and Hemibiotrophic Fungi in Infection." *Cellular Microbiology*. John Wiley &

Sons, Ltd. https://doi.org/10.1111/j.1462-5822.2011.01665.x.

Koren, Sergey, Brian P. Walenz, Konstantin Berlin, Jason R. Miller, Nicholas H. Bergman, and Adam M. Phillippy. 2017. “Canu: Scalable and Accurate Long-Read Assembly via Adaptive κ-Mer Weighting and Repeat Separation.” *Genome Research* 27 (5): 722–36. https://doi.org/10.1101/gr.215087.116.

Kourelis, Jiorgos, and Renier A.L. van der Hoorn. 2018. “Defended to the Nines: 25 Years of Resistance Gene Cloning Identifies Nine Mechanisms for R Protein Function.” *The Plant Cell* 30 (2): 285–99. https://doi.org/10.1105/tpc.17.00579.

Krasileva, Ksenia V., Hans A. Vasquez-Gross, Tyson Howell, Paul Bailey, Francine Paraiso, Leah Clissold, James Simmonds, et al. 2017. “Uncovering Hidden Variation in Polyploid Wheat.” *Proceedings of the National Academy of Sciences of the United States of America* 114 (6): E913–21. https://doi.org/10.1073/pnas.1619268114.

Krasileva, Ksenia V. 2019. “The Role of Transposable Elements and DNA Damage Repair Mechanisms in Gene Duplications and Gene Fusions in Plant Genomes.” *Current Opinion in Plant Biology* 48 (April): 18–25. https://doi.org/10.1016/J.PBI.2019.01.004.

Krattinger, Simon G., Justine Sucher, Liselotte L. Selter, Harsh Chauhan, Bo Zhou, Mingzhi Tang, Narayana M. Upadhyaya, et al. 2016. “The Wheat Durable, Multipathogen Resistance Gene Lr34 Confers Partial Blast Resistance in Rice.” *Plant Biotechnology Journal* 14 (5): 1261–68. https://doi.org/10.1111/pbi.12491.

Krattinger, Simon G, Evans S Lagudah, Wolfgang Spielmeyer, Ravi P Singh, Julio Huerta-Espino, Helen McFadden, Eligio Bossolini, Liselotte L Selter, and Beat Keller. 2009. “A Putative ABC Transporter Confers Durable Resistance to Multiple Fungal Pathogens in Wheat.” *Science* 323 (5919): 1360–63. https://doi.org/10.1126/science.1166453.

Kroj, Thomas, Emilie Chanclud, Corinne Michel-Romiti, Xavier Grand, and Jean Benoit Morel. 2016. “Integration of Decoy Domains Derived from Protein Targets of Pathogen Effectors into Plant Immune Receptors Is Widespread.” *New Phytologist* 210 (2): 618–26. https://doi.org/10.1111/nph.13869.

Kuan, Tung, Yi Zhai, and Wenbo Ma. 2016. “Small RNAs Regulate Plant Responses to Filamentous Pathogens.” *Seminars in Cell and Developmental Biology*. Academic Press. https://doi.org/10.1016/j.semcdb.2016.05.013.

Kuhn, D. M., and M. A. Ghannoum. 2003. “Indoor Mold, Toxigenic Fungi, and Stachybotrys Chartarum: Infectious Disease Perspective.” *Clinical Microbiology Reviews*. American

Society for Microbiology Journals. https://doi.org/10.1128/CMR.16.1.144-172.2003.

Kunkel, Barbara N., and David M. Brooks. 2002. “Cross Talk between Signaling Pathways in Pathogen Defense.” *Current Opinion in Plant Biology*. Elsevier Ltd. https://doi.org/10.1016/S1369-5266(02)00275-3.

Kunze, Gernot, Cyril Zipfel, Silke Robatzek, Karsten Niehaus, Thomas Boller, and Georg Felix. 2004. “The N Terminus of Bacterial Elongation Factor Tu Elicits Innate Immunity in Arabidopsis Plants.” *Plant Cell* 16 (12): 3496–3507. https://doi.org/10.1105/tpc.104.026765.

Kurowski, Tomasz J, and Fady Mohareb. 2020. “Tersect: A Set Theoretical Utility for Exploring Sequence Variant Data.” *Bioinformatics* 36 (3): 934–35. https://doi.org/10.1093/bioinformatics/btz634.

La Concepcion, Juan Carlos De, Marina Franceschetti, Dan Maclean, Ryohei Terauchi, Sophien Kamoun, and Mark J. Banfield. 2019. “Protein Engineering Expands the Effector Recognition Profile of a Rice NLR Immune Receptor.” *ELife* 8 (September). https://doi.org/10.7554/eLife.47713.

la Concepcion, Juan Carlos De, Marina Franceschetti, Abbas Maqbool, Hiromasa Saitoh, Ryohei Terauchi, Sophien Kamoun, and Mark J. Banfield. 2018. “Polymorphic Residues in Rice NLRs Expand Binding and Response to Effectors of the Blast Pathogen.” *Nature Plants* 4 (8): 576–85. https://doi.org/10.1038/s41477-018-0194-x.

Lagudah, Evans S., Simon G. Krattinger, Sybil Herrera-Foessel, Ravi P. Singh, Julio Huerta-Espino, Wolfgang Spielmeyer, Gina Brown-Guedira, Liselotte L. Selter, and Beat Keller. 2009. “Gene-Specific Markers for the Wheat Gene Lr34/Yr18/Pm38 Which Confers Resistance to Multiple Fungal Pathogens.” *Theoretical and Applied Genetics* 119 (5): 889–98. https://doi.org/10.1007/s00122-009-1097-z.

Lakhssassi, Naoufal, Azam Baharlouei, Jonas Meksem, Scott D. Hamilton-Brehm, David A. Lightfoot, Khalid Meksem, and Yanna Liang. 2020. “EMS-Induced Mutagenesis of Clostridium Carboxidivorans for Increased Atmospheric CO2 Reduction Efficiency and Solvent Production.” *Microorganisms* 8 (8): 1239. https://doi.org/10.3390/microorganisms8081239.

Laver, T., J. Harrison, P. A. O’Neill, K. Moore, A. Farbos, K. Paszkiewicz, and D. J. Studholme. 2015. “Assessing the Performance of the Oxford Nanopore Technologies MinION.” *Biomolecular Detection and Quantification* 3 (March): 1–8.

https://doi.org/10.1016/j.bdq.2015.02.001.

Lawrence, Gregory J., Peter N. Dodds, and Jeffrey G. Ellis. 2010. “Transformation of the Flax Rust Fungus, Melampsora Lini: Selection via Silencing of an Avirulence Gene.” *Plant Journal* 61 (2): 364–69. https://doi.org/10.1111/j.1365-313X.2009.04052.x.

Lebkowski, Jane S, Jeffrey H Miller, and M P Calos. 1986. “Determination of DNA Sequence Changes Induced by Ethyl Methanesulfonate in Human Cells, Using a Shuttle Vector System.” *Molecular and Cellular Biology* 6 (5): 1838–42. https://doi.org/10.1128/mcb.6.5.1838.

Lee, Chil Woo, Marina Efetova, Julia C. Engelmann, Robert Kramell, Claus Wasternack, Jutta Ludwig-Müller, Rainer Hedrich, and Rosalia Deeken. 2009. “Agrobacterium Tumefaciens Promotes Tumor Induction by Modulating Pathogen Defense in Arabidopsis Thaliana.” *Plant Cell* 21 (9): 2948–62. https://doi.org/10.1105/tpc.108.064576.

Lee, Wing-Sham, Jason J. Rudd, Kim E. Hammond-Kosack, and Kostya Kanyuka. 2014. “Mycosphaerella Graminicola LysM Effector-Mediated Stealth Pathogenesis Subverts Recognition Through Both CERK1 and CEBiP Homologues in Wheat.” *Molecular Plant-Microbe Interactions®* 27 (3): 236–43. https://doi.org/10.1094/MPMI-07-13-0201-R.

Lehti-Shiu, M D, C Zou, K Hanada, and S H Shiu. 2009. “Evolutionary History and Stress Regulation of Plant Receptor-like Kinase/Pelle Genes.” *Plant Physiol* 150 (1): 12–26. https://doi.org/pp.108.134353 [pii]\r10.1104/pp.108.134353.

Lehti-Shiu, Melissa D., and Shin Han Shiu. 2012. “Diversity, Classification and Function of the Plant Protein Kinase Superfamily.” *Philosophical Transactions of the Royal Society B: Biological Sciences* 367 (1602): 2619–39. https://doi.org/10.1098/rstb.2012.0003.

Lewis, Clare M., Antoine Persoons, Daniel P. Bebber, Rose N. Kigathi, Jens Maintz, Kim Findlay, Vanessa Bueno-Sancho, et al. 2018. “Potential for Re-Emergence of Wheat Stem Rust in the United Kingdom.” *Communications Biology* 1 (1): 13. https://doi.org/10.1038/s42003-018-0013-y.

Leyns, Frederik, Marcel De Cleene, Jean Guy Swings, and Jozef De Ley. 1984. “The Host Range of the Genus Xanthomonas.” *The Botanical Review* 50 (3): 308–56. https://doi.org/10.1007/BF02862635.

Li, Feng, Narayana M. Upadhyaya, Jana Sperschneider, Oadi Matny, Hoa Nguyen-Phuc, Rohit Mago, Castle Raley, et al. 2019. “Emergence of the Ug99 Lineage of the Wheat Stem

Rust Pathogen through Somatic Hybridisation." *Nature Communications* 10 (1): 5068. https://doi.org/10.1038/s41467-019-12927-7.

Li, Heng, and Richard Durbin. 2009. "Fast and Accurate Short Read Alignment with Burrows-Wheeler Transform." *Bioinformatics* 25 (14): 1754–60. https://doi.org/10.1093/bioinformatics/btp324.

Li, Heng, Bob Handsaker, Alec Wysoker, Tim Fennell, Jue Ruan, Nils Homer, Gabor Marth, Goncalo Abecasis, and Richard Durbin. 2009. "The Sequence Alignment/Map Format and SAMtools." *Bioinformatics* 25 (16): 2078–79. https://doi.org/10.1093/bioinformatics/btp352.

Li, Jing, Jing Ding, Wen Zhang, Yuanli Zhang, Ping Tang, Jian Qun Chen, Dacheng Tian, and Sihai Yang. 2010. "Unique Evolutionary Pattern of Numbers of Gramineous NBS-LRR Genes." *Molecular Genetics and Genomics* 283 (5): 427–38. https://doi.org/10.1007/s00438-010-0527-6.

Li, Yuxiang, Meinan Wang, Deven R. See, and Xianming Chen. 2019. "Ethyl-Methanesulfonate Mutagenesis Generated Diverse Isolates of Puccinia Striiformis f. Sp. Tritici, the Wheat Stripe Rust Pathogen." *World Journal of Microbiology and Biotechnology* 35 (2): 28. https://doi.org/10.1007/s11274-019-2600-6.

Li, Yuxiang, Chongjing Xia, Meinan Wang, Chuntao Yin, and Xianming Chen. 2020. "Whole-Genome Sequencing of Puccinia Striiformis f. Sp.Tritici Mutant Isolates Identifies Avirulence Gene Candidates." *BMC Genomics* 21 (1): 1–22. https://doi.org/10.1186/s12864-020-6677-y.

Li, Zhiwu, Ying Liu, and Philip H. Berger. 2005. "Transgene Silencing in Wheat Transformed with the WSMV-CP Gene." *Biotechnology* 4 (1): 62–68. https://doi.org/10.3923/biotech.2005.62.68.

Lin, Xiao, Miles Armstrong, Katie Baker, Doret Wouters, Richard G.F. Visser, Pieter J. Wolters, Ingo Hein, and Vivianne G.A.A. Vleeshouwers. 2020. "RLP/K Enrichment Sequencing; a Novel Method to Identify Receptor-like Protein (RLP) and Receptor-like Kinase (RLK) Genes." *New Phytologist* 227 (4): 1264–76. https://doi.org/10.1111/nph.16608.

Lindgren, P. B., R. C. Peet, and N. J. Panopoulos. 1986. "Gene Cluster of Pseudomonas Syringae Pv. 'phaseolicola' Controls Pathogenicity of Bean Plants and Hypersensitivity on Nonhost Plants." *Journal of Bacteriology* 168 (2): 512–22.

https://doi.org/10.1128/jb.168.2.512-522.1986.

Liu, Changhai, Carsten Pedersen, Torsten Schultz-Larsen, Geziel B. Aguilar, Kenneth Madriz-Ordeñana, Mogens S. Hovmøller, and Hans Thordal-Christensen. 2016. "The Stripe Rust Fungal Effector PEC6 Suppresses Pattern-Triggered Immunity in a Host Species-Independent Manner and Interacts with Adenosine Kinases." *New Phytologist*. https://doi.org/10.1111/nph.14034.

Liu, Jie, Tao Guan, Peijing Zheng, Liyang Chen, Yang Yang, Baoyu Huai, Dan Li, Qing Chang, Lili Huang, and Zhensheng Kang. 2016. "An Extracellular Zn-Only Superoxide Dismutase from Puccinia Striiformis Confers Enhanced Resistance to Host-Derived Oxidative Stress." *Environmental Microbiology* 18 (11): 4118–35. https://doi.org/10.1111/1462-2920.13451.

Liu, Wei, Michele Frick, Rene Huel, Cory L. Nykiforuk, Xiaomin Wang, Denis A. Gaudet, François Eudes, et al. 2014. "The Stripe Rust Resistance Gene Yr10 Encodes an Evolutionary-Conserved and Unique CC-NBS-LRR Sequence in Wheat." *Molecular Plant* 7 (12): 1740–55. https://doi.org/10.1093/mp/ssu112.

Liu, Wenxuan, Matthew Rouse, Bernd Friebe, Yue Jin, Bikram Gill, and Michael O. Pumphrey. 2011. "Discovery and Molecular Mapping of a New Gene Conferring Resistance to Stem Rust, Sr53, Derived from Aegilops Geniculata and Characterization of Spontaneous Translocation Stocks with Reduced Alien Chromatin." *Chromosome Research* 19 (5): 669–82. https://doi.org/10.1007/s10577-011-9226-3.

Liu, Z. H., T. L. Friesen, J. B. Rasmussen, S. Ali, S. W. Meinhardt, and J. D. Faris. 2004. "Quantitative Trait Loci Analysis and Mapping of Seedling Resistance to Stagonospora Nodorum Leaf Blotch in Wheat." *Phytopathology* 94 (10): 1061–67. https://doi.org/10.1094/PHYTO.2004.94.10.1061.

Liu, Zhaohui, and Tim Friesen. 2012. "DAB Staining and Visualization of Hydrogen Peroxide in Wheat Leaves." *BIO-PROTOCOL* 2 (24). https://doi.org/10.21769/BioProtoc.309.

Liu, Zhaohui, Zengcui Zhang, Justin D. Faris, Richard P. Oliver, Robert Syme, Megan C. McDonald, Bruce A. McDonald, et al. 2012. "The Cysteine Rich Necrotrophic Effector SnTox1 Produced by Stagonospora Nodorum Triggers Susceptibility of Wheat Lines Harboring Snn1." Edited by Brett Tyler. *PLoS Pathogens* 8 (1): e1002467. https://doi.org/10.1371/journal.ppat.1002467.

Loegering, W. Q., and E. R. Sears. 1963. "DISTORTED INHERITANCE OF STEM-RUST

RESISTANCE OF TIMSTEIN WHEAT CAUSED BY A POLLEN-KILLING GENE." *Canadian Journal of Genetics and Cytology* 5 (1): 65–72. https://doi.org/10.1139/g63-010.

Lolle, Signe, Danielle Stevens, and Gitta Coaker. 2020. "Plant NLR-Triggered Immunity: From Receptor Activation to Downstream Signaling." *Current Opinion in Immunology* 62 (February): 99–105. https://doi.org/10.1016/j.coi.2019.12.007.

Lorrain, Cécile, Karen Cristine Gonçalves Dos Santos, Hugo Germain, Arnaud Hecker, and Sébastien Duplessis. 2019. "Advances in Understanding Obligate Biotrophy in Rust Fungi." *The New Phytologist* 222 (3): 1190–1206. https://doi.org/10.1111/nph.15641.

Loughman, R., K. Jayasena, and J. Majewski. 2005. "Yield Loss and Fungicide Control of Stem Rust of Wheat." *Australian Journal of Agricultural Research* 56 (1): 91. https://doi.org/10.1071/AR04126.

Lu, Hengyun, Francesca Giordano, and Zemin Ning. 2016. "Oxford Nanopore MinION Sequencing and Genome Assembly." *Genomics, Proteomics & Bioinformatics* 14 (5): 265–79. https://doi.org/10.1016/J.GPB.2016.05.004.

Lu, Shunwen, B. Gillian Turgeon, and Michael C. Edwards. 2015. "A ToxA-like Protein from Cochliobolus Heterostrophus Induces Light-Dependent Leaf Necrosis and Acts as a Virulence Factor with Host Selectivity on Maize." *Fungal Genetics and Biology* 81 (August): 12–24. https://doi.org/10.1016/j.fgb.2015.05.013.

Lu, Xunli, Barbara Kracher, Isabel M.L. Saur, Saskia Bauer, Simon R. Ellwood, Roger Wise, Takashi Yaeno, Takaki Maekawa, and Paul Schulze-Lefert. 2016. "Allelic Barley MLA Immune Receptors Recognize Sequence-Unrelated Avirulence Effectors of the Powdery Mildew Pathogen." *Proceedings of the National Academy of Sciences of the United States of America* 113 (42): E6486–95. https://doi.org/10.1073/pnas.1612947113.

Luig, N. H. 1978. "Mutation Studies in Puccinia Graminis Tritici." In *Proc. 5th Int. Wheat Genetics Symposium*, edited by S Ramanujam, 533–39. New Dehli: Indian Society of Genetics & Plant Breeding.

Luo, Ming, Liqiong Xie, Sambasivam Periyannan, Rohit Mago, Burkhard Steuernagel, Brande Wulff, Brian J. Steffenson, T Lynne Reuber, Evans Lagudah, and Michael Ayliffe. 2018. "Stacking Multiple Stem Rust Resistance Genes at a Single Locus for Durable Resistance in Wheat." In *Plant and Animal Genome XXVI Conference*. San Diego: PAG. https://pag.confex.com/pag/xxvi/meetingapp.cgi/Paper/30410.

Lv, Xuan, Liumeng Pan, Jiaying Wang, Liping Lu, Weilin Yan, Yanye Zhu, Yiwen Xu, Ming Guo,

and Shulin Zhuang. 2017. “Effects of Triazole Fungicides on Androgenic Disruption and CYP3A4 Enzyme Activity.” *Environmental Pollution* 222 (March): 504–12. https://doi.org/10.1016/j.envpol.2016.11.051.

Ma, Li Jun, H. Charlotte Van Der Does, Katherine A. Borkovich, Jeffrey J. Coleman, Marie Josée Daboussi, Antonio Di Pietro, Marie Dufresne, et al. 2010. “Comparative Genomics Reveals Mobile Pathogenicity Chromosomes in Fusarium.” *Nature* 464 (7287): 367–73. https://doi.org/10.1038/nature08850.

Macho, Alberto P., and Cyril Zipfel. 2014. “Plant PRRs and the Activation of Innate Immune Signaling.” *Molecular Cell* 54 (2): 263–72. https://doi.org/10.1016/j.molcel.2014.03.028.

Mackey, David, Youssef Belkhadir, Jose M. Alonso, Joseph R. Ecker, and Jeffery L. Dangl. 2003. “Arabidopsis RIN4 Is a Target of the Type III Virulence Effector AvrRpt2 and Modulates RPS2-Mediated Resistance.” *Cell* 112 (3): 379–89. https://doi.org/10.1016/S0092-8674(03)00040-0.

Mago, Rohit, P. Zhang, H. S. Bariana, D. C. Verlin, U. K. Bansal, J. G. Ellis, and I. S. Dundas. 2009. “Development of Wheat Lines Carrying Stem Rust Resistance Gene Sr39 with Reduced Aegilops Speltoides Chromatin and Simple PCR Markers for Marker-Assisted Selection.” *Theoretical and Applied Genetics* 119 (8): 1441–50. https://doi.org/10.1007/s00122-009-1146-7.

Mago, Rohit, Peng Zhang, Sonia Vautrin, Hana Šimková, Urmil Bansal, Ming Cheng Luo, Matthew Rouse, et al. 2015. “The Wheat Sr50 Gene Reveals Rich Diversity at a Cereal Disease Resistance Locus.” *Nature Plants* 1 (12): 15186. https://doi.org/10.1038/nplants.2015.186.

Maia, Thiago, Jorge L. Badel, Gustavo Marin-Ramirez, Cynthia de M. Rocha, Michelle B. Fernandes, José C. F. da Silva, Gilson M. de Azevedo-Junior, and Sérgio H. Brommonschenkel. 2017. “The *Hemileia Vastatrix* Effector HvEC-016 Suppresses Bacterial Blight Symptoms in Coffee Genotypes with the *S H* 1 Rust Resistance Gene.” *New Phytologist* 213 (3): 1315–29. https://doi.org/10.1111/nph.14334.

Manners, JM, and JL Gay. 1983. “Biochemical Plant Pathoglogy.”

Maqbool, A., H. Saitoh, M. Franceschetti, C. E.M. Stevenson, A. Uemura, H. Kanzaki, S. Kamoun, R. Terauchi, and M. J. Banfield. 2015. “Structural Basis of Pathogen Recognition by an Integrated HMA Domain in a Plant NLR Immune Receptor.” *ELife* 4

(AUGUST2015). https://doi.org/10.7554/eLife.08709.

Marchal, Clemence, Jianping Zhang, Peng Zhang, Paul Fenwick, Burkhard Steuernagel, Nikolai M. Adamski, Lesley Boyd, et al. 2018. “BED-Domain-Containing Immune Receptors Confer Diverse Resistance Spectra to Yellow Rust.” *Nature Plants*. Palgrave Macmillan Ltd. https://doi.org/10.1038/s41477-018-0236-4.

Marone, Daniela, Maria A. Russo, Giovanni Laidò, Anna M. De Leonardis, and Anna M. Mastrangelo. 2013. “Plant Nucleotide Binding Site-Leucine-Rich Repeat (NBS-LRR) Genes: Active Guardians in Host Defense Responses.” *International Journal of Molecular Sciences*. MDPI AG. https://doi.org/10.3390/ijms14047302.

Marshall, Rosalind, Anja Kombrink, Juliet Motteram, Elisa Loza-Reyes, John Lucas, Kim E. Hammond-Kosack, Bart P.H.J. Thomma, and Jason J. Rudd. 2011. “Analysis of Two in Planta Expressed LysM Effector Homologs from the Fungus Mycosphaerella Graminicola Reveals Novel Functional Properties and Varying Contributions to Virulence on Wheat.” *Plant Physiology* 156 (2): 756–69. https://doi.org/10.1104/pp.111.176347.

Martin, F., A. Aerts, D. Ahrén, A. Brun, E. G.J. Danchin, F. Duchaussoy, J. Gibon, et al. 2008. “The Genome of Laccaria Bicolor Provides Insights into Mycorrhizal Symbiosis.” *Nature* 452 (7183): 88–92. https://doi.org/10.1038/nature06556.

Masurekar, Prakash S., Marc P. Kahgan, and Arnold L. Demain. 1972. “Mutagenesis and Enrichment of Auxotrophs in Penicillium Chrysogenum1.” *Applied Microbiology* 24 (6): 995–96. https://doi.org/10.1128/AEM.24.6.995-996.1972.

McDonald, Megan C., Dag Ahren, Steven Simpfendorfer, Andrew Milgate, and Peter S. Solomon. 2018. “The Discovery of the Virulence Gene *ToxA* in the Wheat and Barley Pathogen *Bipolaris Sorokiniana*.” *Molecular Plant Pathology* 19 (2): 432–39. https://doi.org/10.1111/mpp.12535.

McKenna, Aaron, Matthew Hanna, Eric Banks, Andrey Sivachenko, Kristian Cibulskis, Andrew Kernytsky, Kiran Garimella, et al. 2010. “The Genome Analysis Toolkit: A MapReduce Framework for Analyzing next-Generation DNA Sequencing Data.” *Genome Research* 20 (9): 1297–1303. https://doi.org/10.1101/gr.107524.110.

Md Hatta, M. Asyraf, Sanu Arora, Sreya Ghosh, Oadi Matny, Mark A. Smedley, Guotai Yu, Soma Chakraborty, et al. 2020. “The Wheat Sr22 , Sr33 , Sr35 and Sr45 Genes Confer Resistance against Stem Rust in Barley.” *Plant Biotechnology Journal*, August, 1–12.

https://doi.org/10.1111/pbi.13460.

Menardo, Fabrizio, Coraline R. Praz, Thomas Wicker, and Beat Keller. 2017. “Rapid Turnover of Effectors in Grass Powdery Mildew (Blumeria Graminis).” *BMC Evolutionary Biology* 17 (1): 223. https://doi.org/10.1186/s12862-017-1064-2.

Mendgen, K, C Struck, RT Voegele, and M Hahn. 2000. “Biotrophy and Rust Haustoria.” *Physiol Mol Plant Pathol* 56.

Mert, Zafer, Aziz Karakaya, Fazil Düşünceli, Kadir Akan, and Lütfi Çetin. 2012. “Determination of Puccinia Graminis f. Sp. Tritici Races of Wheat in Turkey.” *Turkish Journal of Agriculture and Forestry* 36 (1): 107–20. https://doi.org/10.3906/tar-1010-1278.

Meunier, Etienne, and Petr Broz. 2017. “Evolutionary Convergence and Divergence in NLR Function and Structure.” *Trends in Immunology*. Elsevier Ltd. https://doi.org/10.1016/j.it.2017.04.005.

Meuwissen, T. H.E., B J Hayes, and M E Goddard. 2001. “Prediction of Total Genetic Value Using Genome-Wide Dense Marker Maps.” *Genetics* 157 (4): 1819–29.

Meyer, M., J. A. Cox, M. D.T. Hitchings, L. Burgin, M. C. Hort, D. P. Hodson, and C. A. Gilligan. 2017a. “Quantifying Airborne Dispersal Routes of Pathogens over Continents to Safeguard Global Wheat Supply.” *Nature Plants* 3 (10): 780–86. https://doi.org/10.1038/s41477-017-0017-5.

———. 2017b. “Quantifying Airborne Dispersal Routes of Pathogens over Continents to Safeguard Global Wheat Supply.” *Nature Plants* 3 (10): 780–86. https://doi.org/10.1038/s41477-017-0017-5.

Miller, Marisa E., Ying Zhang, Vahid Omidvar, Jana Sperschneider, Benjamin Schwessinger, Castle Raley, Jonathan M. Palmer, et al. 2018. “De Novo Assembly and Phasing of Dikaryotic Genomes from Two Isolates of Puccinia Coronata f. Sp. Avenae, the Causal Agent of Oat Crown Rust.” *MBio* 9 (1). https://doi.org/10.1128/mBio.01650-17.

Mitema, Alfred, Sheila Okoth, and Suhail M. Rafudeen. 2019. “The Development of a QPCR Assay to Measure Aspergillus Flavus Biomass in Maize and the Use of a Biocontrol Strategy to Limit Aflatoxin Production.” *Toxins* 11 (3). https://doi.org/10.3390/toxins11030179.

Miya, Ayako, Premkumar Albert, Tomonori Shinya, Yoshitake Desaki, Kazuya Ichimura, Ken Shirasu, Yoshihiro Narusaka, Naoto Kawakami, Hanae Kaku, and Naoto Shibuya. 2007.

"CERK1, a LysM Receptor Kinase, Is Essential for Chitin Elicitor Signaling in Arabidopsis." *Proceedings of the National Academy of Sciences of the United States of America* 104 (49): 19613–18. https://doi.org/10.1073/pnas.0705147104.

Moldenhauer, J., B. M. Moerschbacher, and A. J. van der Westhuizen. 2006. "Histological Investigation of Stripe Rust (Puccinia Striiformis f.Sp. Tritici) Development in Resistant and Susceptible Wheat Cultivars." *Plant Pathology* 55 (4): 469–74. https://doi.org/10.1111/j.1365-3059.2006.01385.x.

Möller, Mareike, and Eva H. Stukenbrock. 2017. "Evolution and Genome Architecture in Fungal Plant Pathogens." *Nature Reviews Microbiology* 15 (12): 756–71. https://doi.org/10.1038/nrmicro.2017.76.

Moore, John W., Sybil Herrera-Foessel, Caixia Lan, Wendelin Schnippenkoetter, Michael Ayliffe, Julio Huerta-Espino, Morten Lillemo, et al. 2015. "A Recently Evolved Hexose Transporter Variant Confers Resistance to Multiple Pathogens in Wheat." *Nature Genetics* 47 (12): 1494–98. https://doi.org/10.1038/ng.3439.

Morgan, William, and Sophien Kamoun. 2007. "RXLR Effectors of Plant Pathogenic Oomycetes." *Current Opinion in Microbiology*. Elsevier Current Trends. https://doi.org/10.1016/j.mib.2007.04.005.

Morris, Cindy E., Jay Ram Lamichhane, Ivan Nikolić, Slaviša Stanković, and Benoit Moury. 2019. "The Overlapping Continuum of Host Range among Strains in the Pseudomonas Syringae Complex." *Phytopathology Research* 1 (1): 4. https://doi.org/10.1186/s42483-018-0010-6.

Mu, Xiyan, Tingting Chai, Kai Wang, Lizhen Zhu, Ying Huang, Gongming Shen, Yingren Li, Xuefeng Li, and Chengju Wang. 2016. "The Developmental Effect of Difenoconazole on Zebrafish Embryos: A Mechanism Research." *Environmental Pollution* 212 (May): 18–26. https://doi.org/10.1016/j.envpol.2016.01.035.

Muehlbauer, G. J., J. E. Specht, M. A. Thomas-Compton, P. E. Staswick, and R. L. Bernard. 1988. "Near-Isogenic Lines—A Potential Resource in the Integration of Conventional and Molecular Marker Linkage Maps." *Crop Science* 28 (5): 729–35. https://doi.org/10.2135/cropsci1988.0011183x002800050002x.

Mugford, Sam T., Elaine Barclay, Claire Drurey, Kim C. Findlay, and Saskia A. Hogenhout. 2016. "An Immuno-Suppressive Aphid Saliva Protein Is Delivered into the Cytosol of Plant Mesophyll Cells during Feeding." *Molecular Plant-Microbe Interactions* 29 (11):

854–61. https://doi.org/10.1094/MPMI-08-16-0168-R.

Münch, E. 1930. “Die Stoffbewegung in Der Pflanze. Fischer, Jena.”

Mysore, Kirankumar S., and Choong Min Ryu. 2004. “Nonhost Resistance: How Much Do We Know?” *Trends in Plant Science*. Elsevier Ltd. https://doi.org/10.1016/j.tplants.2003.12.005.

Nagar, Ramawatar, and Benjamin Schwessinger. 2018. “Multi-Step High Purity High Molecular Weight DNA Extraction Protocol from Challenging Fungal Tissues.” *Protocols.Io*, 1–10. https://doi.org/https://doi.org/10.17504/protocols.io.rzkd74w.

Negassa, Asfaw, Bekele Shiferaw, Jawoo Koo, Kai Sonder, Melinda Smale, Hans Joachim Braun, Sika Gbegbelegbe, et al. 2013. “The Potential for Wheat Production in Africa: Analysis of Biophysical Suitability and Economic Profitability.”

Nekrasov, Vladimir, Congmao Wang, Joe Win, Christa Lanz, Detlef Weigel, and Sophien Kamoun. 2017. “Rapid Generation of a Transgene-Free Powdery Mildew Resistant Tomato by Genome Deletion.” *Scientific Reports* 7 (1): 482. https://doi.org/10.1038/s41598-017-00578-x.

Nemri, Adnane, Diane G.O. Saunders, Claire Anderson, Narayana M. Upadhyaya, Joe Win, Gregory J. Lawrence, David A. Jones, Sophien Kamoun, Jeffrey G. Ellis, and Peter N. Dodds. 2014. “The Genome Sequence and Effector Complement of the Flax Rust Pathogen Melampsora Lini.” *Frontiers in Plant Science* 5 (MAR). https://doi.org/10.3389/fpls.2014.00098.

Nester, Eugene W. 2015. “Agrobacterium: Nature’s Genetic Engineer.” *Frontiers in Plant Science* 5 (JAN): 730. https://doi.org/10.3389/fpls.2014.00730.

Newell, S. Y. 2001. “Fungal Biomass and Productivity.” *Methods in Microbiology* 30 (January): 357–72. https://doi.org/10.1016/S0580-9517(01)30053-3.

Niranjana, M. 2017. “Gametocidal Genes of Aegilops: Segregation Distorters in Wheat-Aegilops Wide Hybridization.” *Genome*. Canadian Science Publishing. https://doi.org/10.1139/gen-2017-0023.

Nirmala, Jayaveeramuthu, Tom Drader, Paulraj K. Lawrence, Chuntao Yin, Scot Hulbert, Camille M. Steber, Brian J. Steffenson, Les J. Szabo, Diter Von Wettstein, and Andris Kleinhofs. 2011. “Concerted Action of Two Avirulent Spore Effectors Activates Reaction to Puccinia Graminis 1 (Rpg1)-Mediated Cereal Stem Rust Resistance.” *Proceedings of the National Academy of Sciences of the United States of America* 108 (35): 14676–81.

https://doi.org/10.1073/pnas.1111771108.

Niu, Z., D. L. Klindworth, G. Yu, T. L Friesen, S. Chao, Y. Jin, X. Cai, J.-B. Ohm, J. B. Rasmussen, and Steven S. Xu. 2014. "Development and Characterization of Wheat Lines Carrying Stem Rust Resistance Gene Sr43 Derived from Thinopyrum Ponticum." *Theoretical and Applied Genetics* 127 (4): 969–80. https://doi.org/10.1007/s00122-014-2272-4.

Nordström, Karl J.V., Maria C. Albani, Geo Velikkakam James, Caroline Gutjahr, Benjamin Hartwig, Franziska Turck, Uta Paszkowski, George Coupland, and Korbinian Schneeberger. 2013. "Mutation Identification by Direct Comparison of Whole-Genome Sequencing Data from Mutant and Wild-Type Individuals Using k-Mers." *Nature Biotechnology* 31 (4): 325–30. https://doi.org/10.1038/nbt.2515.

Nowara, Daniela, Patrick Schweizer, Alexandra Gay, Christophe Lacomme, Jane Shaw, Christopher Ridout, Dimitar Douchkov, Götz Hensel, and Jochen Kumlehn. 2010. "HIGS: Host-Induced Gene Silencing in the Obligate Biotrophic Fungal Pathogen Blumeria Graminis." *Plant Cell* 22 (9): 3130–41. https://doi.org/10.1105/tpc.110.077040.

Oerke, E.-C. 2006. "Crop Losses to Pests." *The Journal of Agricultural Science* 144 (01): 31. https://doi.org/10.1017/S0021859605005708.

Ohm, Robin A., Nicolas Feau, Bernard Henrissat, Conrad L. Schoch, Benjamin A. Horwitz, Kerrie W. Barry, Bradford J. Condon, et al. 2012. "Diverse Lifestyles and Strategies of Plant Pathogenesis Encoded in the Genomes of Eighteen Dothideomycetes Fungi." Edited by Alex Andrianopoulos. *PLoS Pathogens* 8 (12): e1003037. https://doi.org/10.1371/journal.ppat.1003037.

Okuyama, Yudai, Hiroyuki Kanzaki, Akira Abe, Kentaro Yoshida, Muluneh Tamiru, Hiromasa Saitoh, Takahiro Fujibe, et al. 2011. "A Multifaceted Genomics Approach Allows the Isolation of the Rice Pia-Blast Resistance Gene Consisting of Two Adjacent NBS-LRR Protein Genes." *Plant Journal* 66 (3): 467–79. https://doi.org/10.1111/j.1365-313X.2011.04502.x.

Oliva, Ricardo, Joe Win, Sylvain Raffaele, Laurence Boutemy, Tolga O. Bozkurt, Angela Chaparro-Garcia, Maria Eugenia Segretin, et al. 2010. "Recent Developments in Effector Biology of Filamentous Plant Pathogens." *Cellular Microbiology* 12 (6): 705–15. https://doi.org/10.1111/j.1462-5822.2010.01471.x.

Olivera Firpo, P D, M Newcomb, K Flath, N. Sommerfeldt-Impe, L J Szabo, M Carter, D G Luster, and Y Jin. 2017. "Characterization of Puccinia Graminis f. Sp. Tritici Isolates

Derived from an Unusual Wheat Stem Rust Outbreak in Germany in 2013." *Plant Pathology* 66 (8): 1258–66. https://doi.org/10.1111/ppa.12674.

Olivera, Pablo D., Matthew N. Rouse, and Yue Jin. 2018. "Identification of New Sources of Resistance to Wheat Stem Rust in Aegilops Spp. in the Tertiary Genepool of Wheat." *Frontiers in Plant Science* 9 (November): 1719. https://doi.org/10.3389/fpls.2018.01719.

Olivera, Pablo D., Zoya Sikharulidze, Rusudan Dumbadze, Les J. Szabo, Maria Newcomb, Ketino Natsarishvili, Matthew N Rouse, Douglas G. Luster, and Yue Jin. 2019. "Presence of a Sexual Population of Puccinia Graminis f. Sp. Tritici in Georgia Provides a Hotspot for Genotypic and Phenotypic Diversity." *Phytopathology* 109 (12): 2152–60. https://doi.org/10.1094/PHYTO-06-19-0186-R.

Olivera, Pablo, Maria Newcomb, Les J. Szabo, Matthew Rouse, Jerry Johnson, Samuel Gale, Douglas G. Luster, et al. 2015a. "Phenotypic and Genotypic Characterization of Race TKTTF of Puccinia Graminis f. Sp. Tritici That Caused a Wheat Stem Rust Epidemic in Southern Ethiopia in 2013-14." *Phytopathology* 105 (7): 917–28. https://doi.org/10.1094/PHYTO-11-14-0302-FI.

Olivera, Pablo, Maria Newcomb, Les J Szabo, Matthew Rouse, Jerry Johnson, Samuel Gale, Douglas G Luster, et al. 2015b. "Phenotypic and Genotypic Characterization of Race TKTTF of Puccinia Graminis f. Sp. Tritici That Caused a Wheat Stem Rust Epidemic in Southern Ethiopia in 2013-14." *Phytopathology* 105 (7): 917–28. https://doi.org/10.1094/PHYTO-11-14-0302-FI.

Ooijen, Gerben van, Gabriele Mayr, Mobien M a Kasiem, Mario Albrecht, Ben J C Cornelissen, and Frank L W Takken. 2008. "Structure-Function Analysis of the NB-ARC Domain of Plant Disease Resistance Proteins." *Journal of Experimental Botany* 59 (6): 1383–97. https://doi.org/10.1093/jxb/ern045.

Oome, Stan, Tom M. Raaymakers, Adriana Cabral, Simon Samwel, Hannah Böhm, Isabell Albert, Thorsten Nürnberger, and Guido Van Den Ackerveken. 2014. "Nep1-like Proteins from Three Kingdoms of Life Act as a Microbe-Associated Molecular Pattern in Arabidopsis." *Proceedings of the National Academy of Sciences of the United States of America* 111 (47): 16955–60. https://doi.org/10.1073/pnas.1410031111.

Oort, A. J.P. 1944. "Onderzoekingen over Stuifbrand - II Overgevoeligheid van Tarwe Voor Stuifbrand (Ustilago Tritici)With a Summary: Hypersensitiviness of Wheat to Loose

Smut." *Tijdschrift Over Plantenziekten* 50 (4): 73–106. https://doi.org/10.1007/BF02056007.

Ortiz, Diana, Karine de Guillen, Stella Cesari, Véronique Chalvon, Jérome Gracy, André Padilla, and Thomas Kroj. 2017. "Recognition of the Magnaporthe Oryzae Effector AVR-Pia by the Decoy Domain of the Rice NLR Immune Receptor RGA5." *The Plant Cell* 29 (1): 156–68. https://doi.org/10.1105/tpc.16.00435.

Orton, E.S., C.M. Lewis, P.E. Davey, G.V. Radhakrishnan, and D.G.O. Saunders. 2019. "Stem Rust (*Puccina Graminis*) Identified on Spring Barley in the UK Adjacent to Infected *Berberis Vulgaris*." *New Disease Reports* 40 (September): 11. https://doi.org/10.5197/j.2044-0588.2019.040.011.

Panwar, Vinay, Brent McCallum, and Guus Bakkeren. 2013a. "Host-Induced Gene Silencing of Wheat Leaf Rust Fungus Puccinia Triticina Pathogenicity Genes Mediated by the Barley Stripe Mosaic Virus." *Plant Molecular Biology* 81 (6): 595–608. https://doi.org/10.1007/s11103-013-0022-7.

———. 2013b. "Endogenous Silencing of Puccinia Triticina Pathogenicity Genes through in Planta-Expressed Sequences Leads to the Suppression of Rust Diseases on Wheat." *Plant Journal* 73 (3): 521–32. https://doi.org/10.1111/tpj.12047.

Pardey, P. G., J. M. Beddow, D. J. Kriticos, T. M. Hurley, R. F. Park, E. Duveiller, R. W. Sutherst, J. J. Burdon, and D. Hodson. 2013. "Right-Sizing Stem-Rust Research." *Science*. American Association for the Advancement of Science. https://doi.org/10.1126/science.122970.

Park, Robert F, and Colin R Wellings. 2012a. "Somatic Hybridization in the Uredinales." https://doi.org/10.1146/annurev-phyto-072910-095405.

———. 2012b. "Somatic Hybridization in the Uredinales." *Annual Review of Phytopathology* 50 (1): 219–39. https://doi.org/10.1146/annurev-phyto-072910-095405.

Parlange, Francis, Guillaume Daverdin, Isabelle Fudal, Marie Line Kuhn, M. H. Balesdent, Françoise Blaise, Bruno Grezes-Besset, and Thierry Rouxel. 2009a. "Leptosphaeria Maculans Avirulence Gene AvrLm4-7 Confers a Dual Recognition Specificity by the Rlm4 and Rlm7 Resistance Genes of Oilseed Rape, and Circumvents Rlm4-Mediated Recognition through a Single Amino Acid Change." *Molecular Microbiology* 71 (4): 851–63. https://doi.org/10.1111/j.1365-2958.2008.06547.x.

Parlange, Francis, Guillaume Daverdin, Isabelle Fudal, Marie Line Kuhn, Marie Hélène

Balesdent, Françoise Blaise, Bruno Grezes-Besset, and Thierry Rouxel. 2009b. "Leptosphaeria Maculans Avirulence Gene AvrLm4-7 Confers a Dual Recognition Specificity by the Rlm4 and Rlm7 Resistance Genes of Oilseed Rape, and Circumvents Rlm4-Mediated Recognition through a Single Amino Acid Change." *Molecular Microbiology* 71 (4): 851–63. https://doi.org/10.1111/j.1365-2958.2008.06547.x.

Patkar, Rajesh N., Peter I. Benke, Ziwei Qu, Yuan Yi Constance Chen, Fan Yang, Sanjay Swarup, and Naweed I. Naqvi. 2015. "A Fungal Monooxygenase-Derived Jasmonate Attenuates Host Innate Immunity." *Nature Chemical Biology* 11 (9): 733–40. https://doi.org/10.1038/nchembio.1885.

Patpour, M., M. S. Hovmøller, A. F. Justesen, M. Newcomb, P. Olivera, Y. Jin, L. J. Szabo, et al. 2016. "Emergence of Virulence to SrTmp in the Ug99 Race Group of Wheat Stem Rust, Puccinia Graminis f. Sp. Tritici, in Africa." *Plant Disease* 100 (2): 522. https://doi.org/10.1094/PDIS-06-15-0668-PDN.

Patpour, Mehran, Annemarie F. Justesen, Asmelash W. Tecle, Mahboobeh Yazdani, Mohsen Yasaie, and Mogens S. Hovmøller. 2020. "First Report of Race TTRTF of Wheat Stem Rust (Puccinia Graminis f. Sp. Tritici) in Eritrea." *Plant Disease* 104 (3). https://doi.org/10.1094/PDIS-10-19-2133-PDN.

Pedersen, Carsten, Emiel Ver Loren van Themaat, Liam J. McGuffin, James C. Abbott, Timothy A. Burgis, Geraint Barton, Laurence V. Bindschedler, et al. 2012. "Structure and Evolution of Barley Powdery Mildew Effector Candidates." *BMC Genomics* 13 (1): 694. https://doi.org/10.1186/1471-2164-13-694.

Peeters, Nemo, Sébastien Carrère, Maria Anisimova, Laure Plener, Anne Claire Cazalé, and Stephane Genin. 2013. "Repertoire, Unified Nomenclature and Evolution of the Type III Effector Gene Set in the Ralstonia Solanacearum Species Complex." *BMC Genomics* 14 (1). https://doi.org/10.1186/1471-2164-14-859.

Periyannan, Sambasivam. 2018. "Sustaining Global Agriculture through Rapid Detection and Deployment of Genetic Resistance to Deadly Crop Diseases." *New Phytologist* 219 (1): 45–51. https://doi.org/10.1111/nph.14928.

Periyannan, Sambasivam, John Moore, Michael Ayliffe, Urmil Bansal, Xiaojing Wang, Li Huang, Karin Deal, et al. 2013a. "The Gene Sr33, an Ortholog of Barley Mla Genes, Encodes Resistance to Wheat Stem Rust Race Ug99." *Science (New York, N.Y.)* 341 (6147): 786–88. https://doi.org/10.1126/science.1239028.

———. 2013b. “The Gene Sr33, an Ortholog of Barley Mla Genes, Encodes Resistance to Wheat Stem Rust Race Ug99.” *Science* 341 (6147): 786–88. https://doi.org/10.1126/science.1239028.

Persoons, Antoine, Emmanuelle Morin, Christine Delaruelle, Thibaut Payen, Fabien Halkett, Pascal Frey, StÃ©phane De Mita, and SÃ©bastien Duplessis. 2014. “Patterns of Genomic Variation in the Poplar Rust Fungus Melampsora Larici-Populina Identify Pathogenesis-Related Factors.” *Frontiers in Plant Science* 5 (SEP): 450. https://doi.org/10.3389/fpls.2014.00450.

Peters Haugrud, Amanda R., Zengcui Zhang, Jonathan K. Richards, Timothy L. Friesen, and Justin D. Faris. 2019. “Genetics of Variable Disease Expression Conferred by Inverse Gene-for-Gene Interactions in the Wheat-Parastagonospora Nodorum Pathosystem.” *Plant Physiology* 180 (1): 420–34. https://doi.org/10.1104/pp.19.00149.

Petre, Benjamin, Cécile Lorrain, Diane G.O. Saunders, Joe Win, Jan Sklenar, Sébastien Duplessis, and Sophien Kamoun. 2016. “Rust Fungal Effectors Mimic Host Transit Peptides to Translocate into Chloroplasts.” *Cellular Microbiology* 18 (4): 453–65. https://doi.org/10.1111/cmi.12530.

Petre, Benjamin, Diane G. O. Saunders, Jan Sklenar, Cécile Lorrain, Ksenia V. Krasileva, Joe Win, Sébastien Duplessis, and Sophien Kamoun. 2016. “Heterologous Expression Screens in Nicotiana Benthamiana Identify a Candidate Effector of the Wheat Yellow Rust Pathogen That Associates with Processing Bodies.” Edited by Mark Gijzen. *PLOS ONE* 11 (2): e0149035. https://doi.org/10.1371/journal.pone.0149035.

Petre, Benjamin, Diane Go Saunders, Jan Sklenar, Cecile Lorrain, Joe Win, Sébastien Duplessis, and Sophien Kamoun. 2015. “Candidate Effector Proteins of the Rust Pathogen Melampsora Larici-Populina Target Diverse Plant Cell Compartments.” *Molecular Plant-Microbe Interactions : MPMI* 28 (6): 689–700. https://doi.org/10.1094/MPMI-01-15-0003-R.

Pingali, Prabhu. 2007. “Westernization of Asian Diets and the Transformation of Food Systems: Implications for Research and Policy.” *Food Policy* 32 (3): 281–98. https://doi.org/10.1016/j.foodpol.2006.08.001.

Plank, J. E. Van der. 1963. *Plant Diseases: Epidemics and Control*. New York, NY: Academic Press Inc.

———. 1968. *Disease Resistance in Plants*. New York, NY: Academic Press Inc.

Plesken, Cecilia, Roland W.S. Weber, Sabrina Rupp, Michaela Leroch, and Matthias Hahn. 2015. “Botrytis Pseudocinerea Is a Significant Pathogen of Several Crop Plants but Susceptible to Displacement by Fungicide-Resistant B. Cinerea Strains.” *Applied and Environmental Microbiology* 81 (20): 7048–56. https://doi.org/10.1128/AEM.01719-15.

Plett, Jonathan M., Yohann Daguerre, Sebastian Wittulsky, Alice Vayssieѐres, Aurelie Deveau, Sarah J. Melton, Annegret Kohler, et al. 2014. “Effector MiSSP7 of the Mutualistic Fungus Laccaria Bicolor Stabilizes the Populus JAZ6 Protein and Represses Jasmonic Acid (JA) Responsive Genes.” *Proceedings of the National Academy of Sciences of the United States of America* 111 (22): 8299–8304. https://doi.org/10.1073/pnas.1322671111.

Pliego, Clara, Daniela Nowara, Giulia Bonciani, Dana M. Gheorghe, Ruo Xu, Priyanka Surana, Ehren Whigham, et al. 2013. “Host-Induced Gene Silencing in Barley Powdery Mildew Reveals a Class of Ribonuclease-Like Effectors.” *Molecular Plant-Microbe Interactions®* 26 (6): 633–42. https://doi.org/10.1094/MPMI-01-13-0005-R.

Plissonneau, Cl??mence, Guillaume Daverdin, B??n??dicte Ollivier, Fran??oise Blaise, Alexandre Degrave, Isabelle Fudal, Thierry Rouxel, and Marie H??l??ne Balesdent. 2016. “A Game of Hide and Seek between Avirulence Genes AvrLm4-7 and AvrLm3 in Leptosphaeria Maculans.” *New Phytologist* 209 (4): 1613–24. https://doi.org/10.1111/nph.13736.

Pollard, E, M D Hooper, and N Moore. 1974. *Hedges (Collins New Naturalist Series)*. London: Collins.

Potter, Thomas L., David D Bosch, and Timothy C Strickland. 2014. “Comparative Assessment of Herbicide and Fungicide Runoff Risk: A Case Study for Peanut Production in the Southern Atlantic Coastal Plain (USA).” *Science of the Total Environment* 490 (August): 1–10. https://doi.org/10.1016/j.scitotenv.2014.04.034.

Pradhanang, P. M., and B. R. Sthapit. 1995. “Effect of Cultivar Mixtures on Yellow Rust Incidence and Grain Yield of Barley in the Hills of Nepal.” *Crop Protection* 14 (4): 331–34. https://doi.org/10.1016/0261-2194(94)00022-Z.

Praz, Coraline R., Salim Bourras, Fansong Zeng, Javier Sánchez-Martín, Fabrizio Menardo, Minfeng Xue, Lijun Yang, et al. 2017. “*AvrPm2* Encodes an RNase-like Avirulence Effector Which Is Conserved in the Two Different Specialized Forms of Wheat and Rye Powdery Mildew Fungus.” *New Phytologist* 213 (3): 1301–14.

https://doi.org/10.1111/nph.14372.

Presti, Libera Lo, Daniel Lanver, Gabriel Schweizer, Shigeyuki Tanaka, Liang Liang, Marie Tollot, Alga Zuccaro, Stefanie Reissmann, and Regine Kahmann. 2015a. “Fungal Effectors and Plant Susceptibility.” *Annual Review of Plant Biology* 66: 513–45. https://doi.org/10.1146/annurev-arplant-043014-114623.

———. 2015b. “Fungal Effectors and Plant Susceptibility.” *Annual Review of Plant Biology* 66 (1): 513–45. https://doi.org/10.1146/annurev-arplant-043014-114623.

Pretorius, Z. A., R. P. Singh, W. W. Wagoire, and T. S. Payne. 2000. “Detection of Virulence to Wheat Stem Rust Resistance Gene Sr31 in Puccinia Graminis . f. Sp. Tritici in Uganda.” *Plant Disease* 84 (2): 203–203. https://doi.org/10.1094/PDIS.2000.84.2.203B.

Pretorius, Z. A., L. J. Szabo, W. H. P. Boshoff, L. Herselman, and B. Visser. 2012. “ First Report of a New TTKSF Race of Wheat Stem Rust (Puccinia Graminis f. Sp. Tritici) in South Africa and Zimbabwe .” *Plant Disease* 96 (4): 590–590. https://doi.org/10.1094/pdis-12-11-1027-pdn.

Pruitt, Rory N., Benjamin Schwessinger, Anna Joe, Nicholas Thomas, Furong Liu, Markus Albert, Michelle R. Robinson, et al. 2015. “The Rice Immune Receptor XA21 Recognizes a Tyrosine-Sulfated Protein from a Gram-Negative Bacterium.” *Science Advances* 1 (6). https://doi.org/10.1126/sciadv.1500245.

Qi, Dong, and Roger W Innes. 2013. “Recent Advances in Plant NLR Structure, Function, Localization, and Signaling.” *Frontiers in Immunology* 0 (October): 348. https://doi.org/10.3389/FIMMU.2013.00348.

Qi, Tiancong, Kyungyong Seong, Daniela P.T. Thomazella, Joonyoung Ryan Kim, Julie Pham, Eunyoung Seo, Myeong Je Cho, Alex Schultink, and Brian J. Staskawicz. 2018. “NRG1 Functions Downstream of EDS1 to Regulate TIR-NLR-Mediated Plant Immunity in Nicotiana Benthamiana.” *Proceedings of the National Academy of Sciences of the United States of America* 115 (46): E10979–87. https://doi.org/10.1073/pnas.1814856115.

Qi, Tuo, Jia Guo, Huan Peng, Peng Liu, Zhensheng Kang, and Jun Guo. 2019. “Host-Induced Gene Silencing: A Powerful Strategy to Control Diseases of Wheat and Barley.” *International Journal of Molecular Sciences*. MDPI AG. https://doi.org/10.3390/ijms20010206.

Quilliam, R. S., and R. C. Shattock. 2003. “Haustoria of Microcyclic Rust Fungi Uromyces

Ficariae and Puccinia Tumida and Other Gall-Forming Species, U. Dactylidis (Macrocyclic) and P. Smyrnii (Demicyclic).” *Plant Pathology* 52 (1): 104–13. https://doi.org/10.1046/j.1365-3059.2003.00800.x.

Ra, Mcintosh. 1983. “Historic Overview of Stem Rust Research.” *Phytopathology*, 1–3.

Radhakrishnan, Guru V., Nicola M. Cook, Vanessa Bueno-Sancho, Clare M. Lewis, Antoine Persoons, Abel Debebe Mitiku, Matthew Heaton, et al. 2019. “MARPLE, a Point-of-Care, Strain-Level Disease Diagnostics and Surveillance Tool for Complex Fungal Pathogens.” *BMC Biology* 17 (1): 1–17. https://doi.org/10.1186/s12915-019-0684-y.

Raffaele, Sylvain, Rhys A. Farrer, Liliana M. Cano, David J. Studholme, Daniel MacLean, Marco Thines, Rays H.Y. Jiang, et al. 2010. “Genome Evolution Following Host Jumps in the Irish Potato Famine Pathogen Lineage.” *Science* 330 (6010): 1540–43. https://doi.org/10.1126/science.1193070.

Raffaele, Sylvain, and Sophien Kamoun. 2012. “Genome Evolution in Filamentous Plant Pathogens: Why Bigger Can Be Better.” *Nature Reviews Microbiology* 10 (6): 417–30. https://doi.org/10.1038/nrmicro2790.

Rafiqi, Maryam, Pamela H.P. Gan, Michael Ravensdale, Gregory J. Lawrence, Jeffrey G. Ellis, David A. Jones, Adrienne R. Hardham, and Peter N. Dodds. 2010. “Internalization of Flax Rust Avirulence Proteins into Flax and Tobacco Cells Can Occur in the Absence of the Pathogen.” *Plant Cell* 22 (6): 2017–32. https://doi.org/10.1105/tpc.109.072983.

Ramachandran, Sowmya R., Chuntao Yin, Joanna Kud, Kiwamu Tanaka, Aaron K. Mahoney, Fangming Xiao, and Scot H. Hulbert. 2017. “Effectors from Wheat Rust Fungi Suppress Multiple Plant Defense Responses.” *Phytopathology* 107 (1): 75–83. https://doi.org/10.1094/PHYTO-02-16-0083-R.

Ray, Deepak K., Nathaniel D. Mueller, Paul C. West, and Jonathan A. Foley. 2013. “Yield Trends Are Insufficient to Double Global Crop Production by 2050.” *PLoS ONE* 8 (6): e66428. https://doi.org/10.1371/journal.pone.0066428.

Redkar, Amey, Elaine Jaeger, and Gunther Doehlemann. 2018. “Visualization of Growth and Morphology of Fungal Hyphae in Planta Using WGA-AF488 and Propidium Iodide Co-Staining.” *BIO-PROTOCOL* 8 (14). https://doi.org/10.21769/bioprotoc.2942.

Redkar, Amey, Alexandra Matei, and Gunther Doehlemann. 2017. “Insights into Host Cell Modulation and Induction of New Cells by the Corn Smut Ustilago Maydis.” *Frontiers in Plant Science* 8 (May): 899. https://doi.org/10.3389/fpls.2017.00899.

REINEKE, GAVIN, BERNADETTE HEINZE, JAN SCHIRAWSKI, HERMANN BUETTNER, REGINE KAHMANN, and CHRISTOPH W. BASSE. 2008. “Indole-3-acetic Acid (IAA) Biosynthesis in the Smut Fungus *Ustilago Maydis* and Its Relevance for Increased IAA Levels in Infected Tissue and Host Tumour Formation.” *Molecular Plant Pathology* 9 (3): 339–55. https://doi.org/10.1111/j.1364-3703.2008.00470.x.

Rep, Martijn, H. Charlotte Van Der Does, Michiel Meijer, Ringo Van Wijk, Petra M. Houterman, Henk L. Dekker, Chris G. De Koster, and Ben J.C. Cornelissen. 2004. “A Small, Cysteine-Rich Protein Secreted by Fusarium Oxysporum during Colonization of Xylem Vessels Is Required for I-3-Mediated Resistance in Tomato.” *Molecular Microbiology* 53 (5): 1373–83. https://doi.org/10.1111/j.1365-2958.2004.04177.x.

Reymond, Philippe, and Edward E. Farmer. 1998. “Jasmonate and Salicylate as Global Signals for Defense Gene Expression.” *Current Opinion in Plant Biology* 1 (5): 404–11. https://doi.org/10.1016/S1369-5266(98)80264-1.

Ribeiro, Orquídea, Frederico Magalhães, Tatiana Q. Aguiar, Marilyn G. Wiebe, Merja Penttilä, and Lucília Domingues. 2013. “Random and Direct Mutagenesis to Enhance Protein Secretion in Ashbya Gossypii.” *Bioengineered* 4 (5): 322. https://doi.org/10.4161/bioe.24653.

Ridout, Christopher J., Pari Skamnioti, Oliver Porritt, Soledad Sacristan, Jonathan D.G. Jones, and James K.M. Brown. 2006a. “Multiple Avirulence Paralogues in Cereal Powdery Mildew Fungi May Contribute to Parasite Fitness and Defeat of Plant Resistance.” *Plant Cell* 18 (9): 2402–14. https://doi.org/10.1105/tpc.106.043307.

Ridout, Christopher J, Pari Skamnioti, Oliver Porritt, Soledad Sacristan, Jonathan D G Jones, and James K M Brown. 2006b. “Multiple Avirulence Paralogues in Cereal Powdery Mildew Fungi May Contribute to Parasite Fitness and Defeat of Plant Resistance.” *The Plant Cell* 18 (9): 2402–14. https://doi.org/10.1105/tpc.106.043307.

Robatzek, Silke, Pascal Bittel, Delphine Chinchilla, Petra Köchner, Georg Felix, Shin Han Shiu, and Thomas Boller. 2007. “Molecular Identification and Characterization of the Tomato Flagellin Receptor LeFLS2, an Orthologue of Arabidopsis FLS2 Exhibiting Characteristically Different Perception Specificities.” *Plant Molecular Biology* 64 (5): 539–47. https://doi.org/10.1007/s11103-007-9173-8.

Rojas-Estevez, Paola, David A. Urbina-Gómez, David A. Ayala-Usma, Natalia Guayazan-Palacios, Maria Fernanda Mideros, Adriana J. Bernal, Martha Cardenas, and Silvia

Restrepo. 2020. “Effector Repertoire of Phytophthora Betacei: In Search of Possible Virulence Factors Responsible for Its Host Specificity.” *Frontiers in Genetics* 11 (June). https://doi.org/10.3389/fgene.2020.00579.

Roman-Reyna, Veronica. 2017. “Understanding Wheat Stripe Rust through Studies on Host and Pathogen Metabolism.” Plant Sciences, Research School of Biology, College of Science, The Australian National University.

Rouse, Matthew N., Eric L. Olson, Bikram S. Gill, Michael O. Pumphrey, and Yue Jin. 2011. “Stem Rust Resistance in Aegilops Tauschii Germplasm.” *Crop Science* 51 (5): 2074–78. https://doi.org/10.2135/cropsci2010.12.0719.

Rouxel, Thierry, and Marie-Hélène Balesdent. 2017. “Life, Death and Rebirth of Avirulence Effectors in a Fungal Pathogen of Brassica Crops, Leptosphaeria Maculans.” *New Phytologist* 214 (2): 526–32. https://doi.org/10.1111/nph.14411.

Rouxel, Thierry, Jonathan Grandaubert, James K. Hane, Claire Hoede, Angela P. Van De Wouw, Arnaud Couloux, Victoria Dominguez, et al. 2011. “Effector Diversification within Compartments of the Leptosphaeria Maculans Genome Affected by Repeat-Induced Point Mutations.” *Nature Communications* 2 (1): 1–10. https://doi.org/10.1038/ncomms1189.

Rsaliyev, A. S., and Sh. S. Rsaliyev. 2018. “Principal Approaches and Achievements in Studying Race Composition of Wheat Stem Rust.” *Vavilovskii Zhurnal Genetiki i Selek-Tsii = Vavilov Journal of Genetics and Breeding* 20 (4): 408–15.

Rudd, Jason J., Kostya Kanyuka, Keywan Hassani-Pak, Mark Derbyshire, Ambrose Andongabo, Jean Devonshire, Artem Lysenko, et al. 2015. “Transcriptome and Metabolite Profiling of the Infection Cycle of Zymoseptoria Tritici on Wheat Reveals a Biphasic Interaction with Plant Immunity Involving Differential Pathogen Chromosomal Contributions and a Variation on the Hemibiotrophic Lifestyle Definition.” *Plant Physiology* 167 (3): 1158–85. https://doi.org/10.1104/pp.114.255927.

Rusttracker. 2017. “Pathotype Tracker - Where Is Ug99.” Rusttracker. 2017. https://rusttracker.cimmyt.org/?page_id=22.

Saintenac, Cyrille, Wing Sham Lee, Florence Cambon, Jason J. Rudd, Robert C. King, William Marande, Stephen J. Powers, et al. 2018. “Wheat Receptor-Kinase-like Protein Stb6 Controls Gene-for-Gene Resistance to Fungal Pathogen Zymoseptoria Tritici.” *Nature Genetics* 50 (3): 368–74. https://doi.org/10.1038/s41588-018-0051-x.

Saintenac, Cyrille, Wenjun Zhang, Andres Salcedo, Matthew N. Rouse, Harold N. Trick, Eduard Akhunov, and Jorge Dubcovsky. 2013a. “Identification of Wheat Gene Sr35 That Confers Resistance to Ug99 Stem Rust Race Group.” *Science* 341 (6147): 783–86. https://doi.org/10.1126/science.1239022.

———. 2013b. “Identification of Wheat Gene Sr35 That Confers Resistance to Ug99 Stem Rust Race Group.” *Science* 341 (AUGUST): 783–86. https://doi.org/10.1126/science.1239022.

Saitoh, Hiromasa, Shizuko Fujisawa, Chikako Mitsuoka, Akiko Ito, Akiko Hirabuchi, Kyoko Ikeda, Hiroki Irieda, et al. 2012. “Large-Scale Gene Disruption in Magnaporthe Oryzae Identifies MC69, a Secreted Protein Required for Infection by Monocot and Dicot Fungal Pathogens.” Edited by Brett Tyler. *PLoS Pathogens* 8 (5): e1002711. https://doi.org/10.1371/journal.ppat.1002711.

Salcedo, Andres, William Rutter, Shichen Wang, Alina Akhunova, Stephen Bolus, Shiaoman Chao, Nickolas Anderson, et al. 2017. “Variation in the AvrSr35 Gene Determines Sr35 Resistance against Wheat Stem Rust Race Ug99.” *Science* 358 (6370): 1604–6. https://doi.org/10.1126/science.aao7294.

Sambasivam, P. K., U. K. Bansal, M. J. Hayden, E. S. Lagudah, and H. S. Bariana. 2008. “Identification of Markers Linked with Stem Rust Resistance Genes Sr33 and Sr45.” In *The 11th International Wheat Genetics Symposium*, 20–22. http://www.fao.org/newsroom/en/news/2008/10008.

Sánchez-Martín, Javier, Burkhard Steuernagel, Sreya Ghosh, Gerhard Herren, Severine Hurni, Nikolai Adamski, Jan Vrána, et al. 2016a. “Rapid Gene Isolation in Barley and Wheat by Mutant Chromosome Sequencing.” *Genome Biology* 17 (1). https://doi.org/10.1186/s13059-016-1082-1.

———. 2016b. “Rapid Gene Isolation in Barley and Wheat by Mutant Chromosome Sequencing.” *Genome Biology* 17 (1): 221. https://doi.org/10.1186/s13059-016-1082-1.

Sánchez-Vallet, Andrea, Hui Tian, Luis Rodriguez-Moreno, Dirk Jan Valkenburg, Raspudin Saleem-Batcha, Stephan Wawra, Anja Kombrink, et al. 2020. “A Secreted LysM Effector Protects Fungal Hyphae through Chitin-Dependent Homodimer Polymerization.” Edited by Hui-Shan Guo. *PLoS Pathogens* 16 (6 June): e1008652. https://doi.org/10.1371/journal.ppat.1008652.

Sattelmacher, B. 2001. “The Apoplast and Its Significance for Plant Mineral Nutrition.” *New*

Phytologist. John Wiley & Sons, Ltd. https://doi.org/10.1046/j.1469-8137.2001.00034.x.

Saunders, Diane G O, Joe Win, Liliana M. Cano, Les J. Szabo, Sophien Kamoun, and Sylvain Raffaele. 2012. "Using Hierarchical Clustering of Secreted Protein Families to Classify and Rank Candidate Effectors of Rust Fungi." Edited by Jason E. Stajich. *PLoS ONE* 7 (1): e29847. https://doi.org/10.1371/journal.pone.0029847.

Saur, Isabel M.L., Saskia Bauer, Barbara Kracher, Xunli Lu, Lamprinos Franzeskakis, Marion C. Müller, Björn Sabelleck, et al. 2019. "Multiple Pairs of Allelic MLA Immune Receptor-Powdery Mildew AVR a Effectors Argue for a Direct Recognition Mechanism." *ELife* 8 (February). https://doi.org/10.7554/eLife.44471.

Saur, Isabel M.L., Saskia Bauer, Xunli Lu, and Paul Schulze-Lefert. 2019. "A Cell Death Assay in Barley and Wheat Protoplasts for Identification and Validation of Matching Pathogen AVR Effector and Plant NLR Immune Receptors." *Plant Methods* 15 (1): 118. https://doi.org/10.1186/s13007-019-0502-0.

Savary, Serge, Laetitia Willocquet, Sarah Jane Pethybridge, Paul Esker, Neil McRoberts, and Andy Nelson. 2019a. "The Global Burden of Pathogens and Pests on Major Food Crops." *Nature Ecology and Evolution* 3 (3): 430–39. https://doi.org/10.1038/s41559-018-0793-y.

———. 2019b. "The Global Burden of Pathogens and Pests on Major Food Crops." *Nature Ecology and Evolution* 3 (3): 430–39. https://doi.org/10.1038/s41559-018-0793-y.

Schaefer, Luisa Katharina, Francis Parlange, Gabriele Buchmann, Esther Jung, Andreas Wehrli, Gerhard Herren, Marion Claudia Müller, et al. 2020. "Cross-Kingdom RNAi of Pathogen Effectors Leads to Quantitative Adult Plant Resistance in Wheat." *Frontiers in Plant Science* 0 (March): 253. https://doi.org/10.3389/FPLS.2020.00253.

Schindelin, Johannes, Ignacio Arganda-Carreras, Erwin Frise, Verena Kaynig, Mark Longair, Tobias Pietzsch, Stephan Preibisch, et al. 2012. "Fiji: An Open-Source Platform for Biological-Image Analysis." *Nature Methods*. Nature Publishing Group. https://doi.org/10.1038/nmeth.2019.

Schmidt, Sarah M., Petra M. Houterman, Ines Schreiver, Lisong Ma, Stefan Amyotte, Biju Chellappan, Sjef Boeren, Frank L W Takken, and Martijn Rep. 2013. "MITEs in the Promoters of Effector Genes Allow Prediction of Novel Virulence Genes in Fusarium Oxysporum." *BMC Genomics* 14 (1): 119. https://doi.org/10.1186/1471-2164-14-119.

Schnabel, Guido, and Alan L Jones. 2000. “The 14α − Demethylase (CYP51A1) Gene Is Overexpressed in Venturia Inaequalis Strains Resistant to Myclobutanil.” *Phytopathology* 91 (1): 102–10. https://doi.org/10.1094/PHYTO.2001.91.1.102.

Schornack, Sebastian, Mireille Van Damme, Tolga O. Bozkurt, Liliana M. Cano, Matthew Smoker, Marco Thines, Elodie Gaulin, Sophien Kamoun, and Edgar Huitema. 2010. “Ancient Class of Translocated Oomycete Effectors Targets the Host Nucleus.” *Proceedings of the National Academy of Sciences of the United States of America* 107 (40): 17421–26. https://doi.org/10.1073/pnas.1008491107.

Schumann, G. L., and K.J. Leonard. 2000. “Stem Rust of Wheat (Black Rust).” *The Plant Health Instructor* 58 (April): 1–12. https://doi.org/10.1094/PHI-I-2000-0721-01.

Schwessinger, Benjamin, Jana Sperschneider, William S. Cuddy, Diana P. Garnica, Marisa E. Miller, Jennifer M. Taylor, Peter N. Dodds, Melania Figueroa, Robert F. Park, and John P. Rathjen. 2018. “A Near-Complete Haplotype-Phased Genome of the Dikaryotic Wheat Stripe Rust Fungus Puccinia Striiformis f. Sp. Tritici Reveals High Interhaplotype Diversity.” *MBio* 9 (1): 2020. https://doi.org/10.1128/mBio.02275-17.

Schwessinger, Benjamin, and Cyril Zipfel. 2008. “News from the Frontline: Recent Insights into PAMP-Triggered Immunity in Plants.” *Current Opinion in Plant Biology* 11 (4): 389–95. https://doi.org/10.1016/j.pbi.2008.06.001.

Segretin, María Eugenia, Marina Pais, Marina Franceschetti, Angela Chaparro-Garcia, Jorunn I.B. Bos, Mark J Banfield, and Sophien Kamoun. 2014. “Single Amino Acid Mutations in the Potato Immune Receptor R3a Expand Response to Phytophthora Effectors.” *Molecular Plant-Microbe Interactions* 27 (7): 624–37. https://doi.org/10.1094/MPMI-02-14-0040-R.

Selin, Carrie, Teresa R. de Kievit, Mark F. Belmonte, and W. G Dilantha Fernando. 2016. *Elucidating the Role of Effectors in Plant-Fungal Interactions: Progress and Challenges*. *Frontiers in Microbiology*. Vol. 7. Frontiers Media SA. https://doi.org/10.3389/fmicb.2016.00600.

Sella, Luca, Katia Gazzetti, Franco Faoro, Silvana Odorizzi, Renato D’Ovidio, Wilhelm Schäfer, and Francesco Favaron. 2013. “A Fusarium Graminearum Xylanase Expressed during Wheat Infection Is a Necrotizing Factor but Is Not Essential for Virulence.” *Plant Physiology and Biochemistry* 64 (March): 1–10. https://doi.org/10.1016/j.plaphy.2012.12.008.

Seto, Derek, Noushin Koulena, Timothy Lo, Alexandra Menna, David S. Guttman, and Darrell Desveaux. 2017a. “Expanded Type III Effector Recognition by the ZAR1 NLR Protein Using ZED1-Related Kinases.” *Nature Plants* 3 (4): 1–4. https://doi.org/10.1038/nplants.2017.27.

———. 2017b. “Expanded Type III Effector Recognition by the ZAR1 NLR Protein Using ZED1-Related Kinases.” *Nature Plants* 3 (4): 17027. https://doi.org/10.1038/nplants.2017.27.

Shao, Zhu Qing, Yan Mei Zhang, Yue Yu Hang, Jia Yu Xue, Guang Can Zhou, Ping Wu, Xiao Yi Wu, et al. 2014a. “Long-Term Evolution of Nucleotide-Binding Site-Leucine-Rich Repeat Genes: Understanding Gained from and beyond the Legume Family.” *Plant Physiology* 166 (1): 217–34. https://doi.org/10.1104/pp.114.243626.

———. 2014b. “Long-Term Evolution of Nucleotide-Binding Site-Leucine-Rich Repeat Genes: Understanding Gained from and beyond the Legume Family.” *Plant Physiology* 166 (1): 217–34. https://doi.org/10.1104/pp.114.243626.

Sharma Poudel, Roshan, Abdullah F. Al-Hashel, Thomas Gross, Patrick Gross, and Robert Brueggeman. 2018. “Pyramiding Rpg4- And Rpg1-Mediated Stem Rust Resistance in Barley Requires the Rrr1 Gene for Both to Function.” *Frontiers in Plant Science* 871 (December): 1789. https://doi.org/10.3389/fpls.2018.01789.

Shen, Qing, Yingyao Liu, and Naweed I Naqvi. 2018. “Fungal Effectors at the Crossroads of Phytohormone Signaling.” *Current Opinion in Microbiology* 46 (December): 1–6. https://doi.org/10.1016/j.mib.2018.01.006.

Shi, Gongjun, Zengcui Zhang, Timothy L. Friesen, Dina Raats, Tzion Fahima, Robert S. Brueggeman, Shunwen Lu, et al. 2016. “The Hijacking of a Receptor Kinase–Driven Pathway by a Wheat Fungal Pathogen Leads to Disease.” *Science Advances* 2 (10): e1600822. https://doi.org/10.1126/sciadv.1600822.

Shirasawa, Kenta, Hideki Hirakawa, Tsukasa Nunome, Satoshi Tabata, and Sachiko Isobe. 2016. “Genome-Wide Survey of Artificial Mutations Induced by Ethyl Methanesulfonate and Gamma Rays in Tomato.” *Plant Biotechnology Journal* 14 (1): 51–60. https://doi.org/10.1111/pbi.12348.

Sierotzki, Helge, Sandro Parisi, Ute Steinfeld, Isabel Tenzer, Sylvie Poirey, and Ulrich Gisi. 2000. “Mode of Resistance to Respiration Inhibitors at the Cytochrome Bc1 Enzyme Complex of Mycosphaerella Fijiensis Field Isolates.” *Pest Management Science* 56 (10): 833–41. https://doi.org/10.1002/1526-4998(200010)56:10<833::AID-PS200>3.0.CO;2-

Q.

Singh, G., J. Nirmala, and M. N. Rouse. 2013. “EMS Mutagenesis of Avirulent Puccinia Graminis f. Sp. Tritici Urediniospores.” In *BGRI 2013 Technical Workshop*, 112. New Dehli.

Singh, R. P., J. Huerta-Espino, and A. P. Roelfs. 2002. “The Wheat Rusts.” *Bread Wheat Improvement and Production*, 1–13. http://www.fao.org/docrep/006/y4011e/y4011e0g.htm.

Singh, Ravi P., David P. Hodson, Julio Huerta-Espino, Yue Jin, Peter Njau, Ruth Wanyera, Sybil A. Herrera-Foessel, and Richard W. Ward. 2008. “Will Stem Rust Destroy the World’s Wheat Crop?” In *Advances in Agronomy*, 98:271–309. Academic Press. https://doi.org/10.1016/S0065-2113(08)00205-8.

Singh, Ravi P., David P. Hodson, Yue Jin, Evans S. Lagudah, Michael A. Ayliffe, Sridhar Bhavani, Matthew N. Rouse, et al. 2015. “Emergence and Spread of New Races of Wheat Stem Rust Fungus: Continued Threat to Food Security and Prospects of Genetic Control.” *Phytopathology* 105 (7): 872–84. https://doi.org/10.1094/PHYTO-01-15-0030-FI.

Singh, Ravi P, David P Hodson, Julio Huerta-Espino, Yue Jin, Sridhar Bhavani, Peter Njau, Sybil Herrera-Foessel, Pawan K Singh, Sukhwinder Singh, and Velu Govindan. 2011. “The Emergence of Ug99 Races of the Stem Rust Fungus Is a Threat to World Wheat Production.” *Annual Review of Phytopathology* 49 (April): 465–81. https://doi.org/10.1146/annurev-phyto-072910-095423.

Singh, Ravi P, David P Hodson, Yue Jin, Julio Huerta-Espino, Miriam G Kinyua, Ruth Wanyera, Peter Njau, and Rick W Ward. 2006. “Current Status, Likely Migration and Strategies to Mitigate the Threat to Wheat Production from Race Ug99 (TTKS) of Stem Rust Pathogen.” *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*. https://doi.org/10.1079/PAVSNNR20061054.

Slageren, M. W. Van. 1994. “Wild Wheats: A Monograph of Aegilops L. and Amblyopyrum (Jaub. & Spach) Eig (Poaceae).” *Wageningen Agricultural University Papers* 94–7. https://www.cabdirect.org/cabdirect/abstract/19951602046.

Snelders, Nick C., Hanna Rovenich, Gabriella C. Petti, Mercedes Rocafort, Grardy C. M. van den Berg, Julia A. Vorholt, Jeroen R. Mesters, Michael F. Seidl, Reindert Nijland, and Bart P. H. J. Thomma. 2020. “Microbiome Manipulation by a Soil-Borne Fungal Plant

Pathogen Using Effector Proteins." *Nature Plants*, November, 1–10. https://doi.org/10.1038/s41477-020-00799-5.

Sohn, Jürgen, Ralf T. Voegele, Kurt Mendgen, and Matthias Hahn. 2000. "High Level Activation of Vitamin B1 Biosynthesis Genes in Haustoria of the Rust Fungus Uromyces Fabae." *Molecular Plant-Microbe Interactions* 13 (6): 629–36. https://doi.org/10.1094/MPMI.2000.13.6.629.

Solanki, Shyam, Gazala Ameen, Pawel Borowicz, and Robert S. Brueggeman. 2019. "Shedding Light on Penetration of Cereal Host Stomata by Wheat Stem Rust Using Improved Methodology." *Scientific Reports* 9 (1): 1–13. https://doi.org/10.1038/s41598-019-44280-6.

Spanu, Pietro D. 2017. "Cereal Immunity against Powdery Mildews Targets RNase-Like Proteins Associated with Haustoria (RALPH) Effectors Evolved from a Common Ancestral Gene." *New Phytologist* 213 (3): 969–71. https://doi.org/10.1111/nph.14386.

Sperschneider, Jana, Ann Maree Catanzariti, Kathleen Deboer, Benjamin Petre, Donald M. Gardiner, Karam B. Singh, Peter N. Dodds, and Jennifer M. Taylor. 2017. "LOCALIZER: Subcellular Localization Prediction of Both Plant and Effector Proteins in the Plant Cell." *Scientific Reports* 7 (1): 44598. https://doi.org/10.1038/srep44598.

Sperschneider, Jana, Peter N. Dodds, Jennifer M. Taylor, and Sébastien Duplessis. 2017. "Computational Methods for Predicting Effectors in Rust Pathogens." In *Methods in Molecular Biology*, 1659:73–83. Humana Press Inc. https://doi.org/10.1007/978-1-4939-7249-4_7.

Sperschneider, Jana, Donald M. Gardiner, Peter N. Dodds, Francesco Tini, Lorenzo Covarelli, Karam B. Singh, John M. Manners, and Jennifer M. Taylor. 2016a. "EffectorP: Predicting Fungal Effector Proteins from Secretomes Using Machine Learning." *New Phytologist* 210 (2): 743–61. https://doi.org/10.1111/nph.13794.

———. 2016b. "EffectorP: Predicting Fungal Effector Proteins from Secretomes Using Machine Learning." *New Phytologist* 210 (2): 743–61. https://doi.org/10.1111/nph.13794.

Staal, Jens, Maria Kaliff, Svante Bohman, and Christina Dixelius. 2006. "Transgressive Segregation Reveals Two Arabidopsis TIR-NB-LRR Resistance Genes Effective against *Leptosphaeria Maculans* , Causal Agent of Blackleg Disease." *The Plant Journal* 46 (2): 218–30. https://doi.org/10.1111/j.1365-313X.2006.02688.x.

Stakman, E C. 1923. “Barberry Eradication Prevents Black Rust in Western Europe.” *United States Department of Agriculture, Department Circular 269*, 1–15.

Stakman, E C, D M Stewart, and W Q Loegering. 1962. “Identification of Physiologic Races of Puccinia Graminis Var. Tritici.” *USDA ARS E-617*. Washington DC: U.S. Gov. Print. Office. https://naldc.nal.usda.gov/download/CAT10243018/PDF.

Stakman, Elvin Charles, and M N Levine. 1922. “The Determination of Biologic Forms of Puccinia Graminis on Triticum Spp.” *The University of Minnesota Agricultural Experiment Station Technical Bulletin* 8: 1–10.

Stanke, Mario, and Burkhard Morgenstern. 2005. “AUGUSTUS: A Web Server for Gene Prediction in Eukaryotes That Allows User-Defined Constraints.” *Nucleic Acids Research* 33 (SUPPL. 2). https://doi.org/10.1093/nar/gki458.

Staskawicz, B. J., D. Dahlbeck, and N. T. Keen. 1984. “Cloned Avirulence Gene of *Pseudomonas Syringae Pv. Glycinea* Determines Race-Specific Incompatibility on Glycine Max (L.) Merr.” *Proceedings of the National Academy of Sciences of the United States of America* 81 (19): 6024–28. https://doi.org/10.1073/pnas.81.19.6024.

Stassen, Joost H.M., Michael F. Seidl, Pim W.J. Vergeer, Isaäc J. Nijman, Berend Snel, Edwin Cuppen, and Guido Van Den Ackerveken. 2012. “Effector Identification in the Lettuce Downy Mildew Bremia Lactucae by Massively Parallel Transcriptome Sequencing.” *Molecular Plant Pathology* 13 (7): 719–31. https://doi.org/10.1111/j.1364-3703.2011.00780.x.

Stergiopoulos, Ioannis, Jérôme Collemare, Rahim Mehrabi, and Pierre J.G.M. De Wit. 2013. “Phytotoxic Secondary Metabolites and Peptides Produced by Plant Pathogenic Dothideomycete Fungi.” *FEMS Microbiology Reviews*. Oxford Academic. https://doi.org/10.1111/j.1574-6976.2012.00349.x.

Stergiopoulos, Ioannis, and Pierre J.G.M. De Wit. 2009. “Fungal Effector Proteins.” *Annual Review of Phytopathology* 47 (1): 233–63. https://doi.org/10.1146/annurev.phyto.112408.132637.

Steuernagel, Burkhard, Florian Jupe, Kamil Witek, Jonathan D G Jones, and Brande B H Wulff. 2015. “NLR-Parser: Rapid Annotation of Plant NLR Complements.” *Bioinformatics* 31 (10): 1665–67. https://doi.org/10.1093/bioinformatics/btv005.

Steuernagel, Burkhard, Sambasivam K Periyannan, Inmaculada Hernández-Pinzón, Kamil Witek, Matthew N Rouse, Guotai Yu, Asyraf Hatta, et al. 2016. “Rapid Cloning of

Disease-Resistance Genes in Plants Using Mutagenesis and Sequence Capture." *Nature Biotechnology* 34 (6): 652–55. https://doi.org/10.1038/nbt.3543.

Steuernagel, Burkhard, Jan Vrána, Miroslava Karafiátová, Brande B.H. Wulff, and Jaroslav Doležel. 2017. "Rapid Gene Isolation Using MutChromSeq." In *Methods in Molecular Biology*, 1659:231–43. https://doi.org/10.1007/978-1-4939-7249-4_20.

Steuernagel, Burkhard, Kamil Witek, Simon G Krattinger, Ricardo H. Ramirez-Gonzalez, Henk-jan Schoonbeek, Guotai Yu, Erin Baggs, et al. 2020. "The NLR-Annotator Tool Enables Annotation of the Intracellular Immune Receptor Repertoire." *Plant Physiology*, pp.01273.2019. https://doi.org/10.1104/pp.19.01273.

Storer, Nicholas P., Gary D. Thompson, and Graham P. Head. 2012. "Application of Pyramided Traits against Lepidoptera in Insect Resistance Management for Bt Crops." *GM Crops & Food* 3 (3): 154–62. https://doi.org/10.4161/gmcr.20945.

Stubbs, R W. 1968. "Artificial Mutation in the Study of the Relationship between Races of Yellow Rust of Wheat." In *Proc. Eur. and Med. Cereal Rusts Conf.* Lisbon.

Tadesse, Wuletaw, Miguel Sanchez-Garcia, Solomon Gizaw Assefa, Ahmed Amri, Zewdie Bishaw, Francis C Ogbonnaya, and Michael Baum. 2019. "Genetic Gains in Wheat Breeding and Its Role in Feeding the World." *Crop Breeding, Genetics and Genomics*. https://doi.org/10.20900/cbgg20190005.

Takken, Frank, and Martijn Rep. 2010. "The Arms Race between Tomato and Fusarium Oxysporum." *Molecular Plant Pathology*. Mol Plant Pathol. https://doi.org/10.1111/j.1364-3703.2009.00605.x.

Talhinhas, Pedro, Helena G. Azinheira, Bruno Vieira, Andreia Loureiro, SÃ-lvia Tavares, Dora Batista, Emmanuelle Morin, et al. 2014. "Overview of the Functional Virulent Genome of the Coffee Leaf Rust Pathogen Hemileia Vastatrix with an Emphasis on Early Stages of Infection." *Frontiers in Plant Science* 5 (MAR): 88. https://doi.org/10.3389/fpls.2014.00088.

Tamborski, Janina, and Ksenia V. Krasileva. 2020. "Evolution of Plant NLRs: From Natural History to Precise Modifications." *Annual Review of Plant Biology*. Annual Reviews Inc. https://doi.org/10.1146/annurev-arplant-081519-035901.

Tameling, Wladimir I.L., Sandra D.J. Elzinga, Patricia S. Darmin, Jack H. Vossen, Frank L.W. Takken, Michel A. Haring, and Ben J.C. Cornelissen. 2002. "The Tomato R Gene Products I-2 and Mi-1 Are Functional ATP Binding Proteins with ATPase Activity." *Plant*

Cell 14 (11): 2929–39. https://doi.org/10.1105/tpc.005793.

Tanaka, Eiji, Hironori Koga, Mari Mori, and Masashi Mori. 2011. "Auxin Production by the Rice Blast Fungus and Its Localization in Host Tissue." *Journal of Phytopathology* 159 (7–8): 522–30. https://doi.org/10.1111/j.1439-0434.2011.01799.x.

Tang, Chunlei, Xiaojie Wang, Yulin Cheng, Minjie Liu, Mengxin Zhao, Jinping Wei, and Zhensheng Kang. 2015. "New Insights in the Battle between Wheat and Puccinia Striiformis." *Frontiers of Agricultural Science and Engineering* 2 (2): 101–14. https://doi.org/10.15302/J-FASE-2015068.

Tang, Chunlei, Qiang Xu, Mengxin Zhao, Xiaojie Wang, and Zhensheng Kang. 2018. "Understanding the Lifestyles and Pathogenicity Mechanisms of Obligate Biotrophic Fungi in Wheat: The Emerging Genomics Era." *The Crop Journal* 6 (1): 60–67. https://doi.org/10.1016/j.cj.2017.11.003.

Tang, Dingzhong, Guoxun Wang, and Jian Min Zhou. 2017. "Receptor Kinases in Plant-Pathogen Interactions: More than Pattern Recognition." *Plant Cell*. American Society of Plant Biologists. https://doi.org/10.1105/tpc.16.00891.

Tang, Xiaoyan, Reid D. Frederick, Jianmin Zhou, Dennis A. Halterman, Yulin Jia, and Gregory B. Martin. 1996. "Initiation of Plant Disease Resistance by Physical Interaction of AvrPto and Pto Kinase." *Science* 274 (5295): 2060–63. https://doi.org/10.1126/science.274.5295.2060.

Teo, C., and E. P. Baker. 1966. "Mutants of Puccinia Graminis Avenae Induced by Ethyl Methane Sulphonate." *Nature* 209 (2): 632–33. https://doi.org/10.1038/209632b0.

Terefe, T., Z. A. Pretorius, B. Visser, and W. H. P. Boshoff. 2019. " First Report of Puccinia Graminis f. Sp. Tritici Race PTKSK, a Variant of Wheat Stem Rust Race Ug99, in South Africa ." *Plant Disease* 103 (6): 1421–1421. https://doi.org/10.1094/pdis-11-18-1911-pdn.

Thara, Venkatappa K., John P. Fellers, and Jian Min Zhou. 2003. "In Planta Induced Genes of Puccinia Triticina." *Molecular Plant Pathology* 4 (1): 51–56. https://doi.org/10.1046/j.1364-3703.2003.00142.x.

Thind, Anupriya Kaur, Thomas Wicker, Hana Šimková, Dario Fossati, Odile Moullet, Cécile Brabant, Jan Vrána, Jaroslav Doležel, and Simon G. Krattinger. 2017. "Rapid Cloning of Genes in Hexaploid Wheat Using Cultivar-Specific Long-Range Chromosome Assembly." *Nature Biotechnology* 35 (8): 793–96. https://doi.org/10.1038/nbt.3877.

Thomas, Colwyn M., David A. Jones, Martin Parniske, Kate Harrison, Peter Balint-Kurti, Kostas Hatzixanthis, and Jonathan Dallas Jones. 1997. “Characterization of the Tomato Cf-4 Gene for Resistance to Cladosporium Fulvum Identifies Sequences That Determine Recognitional Specificity in Cf-4 and Cf-9.” *Plant Cell* 9 (12): 2209–24. https://doi.org/10.1105/tpc.9.12.2209.

Thordal-Christensen, Hans, Ziguo Zhang, Yangdou Wei, and David B. Collinge. 1997a. “Subcellular Localization of H2O2 in Plants. H2O2 Accumulation in Papillae and Hypersensitive Response during the Barley-Powdery Mildew Interaction.” *Plant Journal* 11 (6): 1187–94. https://doi.org/10.1046/j.1365-313X.1997.11061187.x.

———. 1997b. “Subcellular Localization of H2O2 in Plants. H2O2 Accumulation in Papillae and Hypersensitive Response during the Barley-Powdery Mildew Interaction.” *Plant Journal* 11 (6): 1187–94. https://doi.org/10.1046/j.1365-313X.1997.11061187.x.

Tian, Miaoying, Joe Win, Jing Song, Renier Van Der Hoorn, Esther Van Der Knaap, and Sophien Kamoun. 2007. “A Phytophthora Infestans Cystatin-like Protein Targets a Novel Tomato Papain-like Apoplastic Protease.” *Plant Physiology* 143 (1): 364–77. https://doi.org/10.1104/pp.106.090050.

Tian, Yuan, Gangming Zhan, Xianming Chen, Angkana Tungruentragoon, Xia Lu, Jie Zhao, Lili Huang, and Zhensheng Kang. 2016. “Virulence and Simple Sequence Repeat Marker Segregation in a *Puccinia Striiformis* f. Sp. *Tritici* Population Produced by Selfing a Chinese Isolate on *Berberis Shensiana*.” *Phytopathology®* 106 (2): 185–91. https://doi.org/10.1094/PHYTO-07-15-0162-R.

Ting, Jenny P.Y., Ruth C. Lovering, Emad S. Alnemri, John Bertin, Jeremy M. Boss, Beckley K. Davis, Richard A. Flavell, et al. 2008. “The NLR Gene Family: A Standard Nomenclature.” *Immunity*. Cell Press. https://doi.org/10.1016/j.immuni.2008.02.005.

Tohge, Takayuki, Mutsumi Watanabe, Rainer Hoefgen, and Alisdair R. Fernie. 2013. “Shikimate and Phenylalanine Biosynthesis in the Green Lineage.” *Frontiers in Plant Science* 4 (MAR): 62. https://doi.org/10.3389/fpls.2013.00062.

Torabi, M., and J. G. Manners. 1989. “Appressorium Formation of Puccinia Recondita on Susceptible and Resistant Wheat Cultivars.” *Mycological Research* 92 (4): 440–44. https://doi.org/10.1016/S0953-7562(89)80188-1.

Tripathi, Jaindra N., Jim Lorenzen, Ofir Bahar, Pamela Ronald, and Leena Tripathi. 2014. “Transgenic Expression of the Rice Xa21 Pattern-Recognition Receptor in Banana (Musa

Sp.) Confers Resistance to Xanthomonas Campestris Pv. Musacearum." *Plant Biotechnology Journal* 12 (6): 663–73. https://doi.org/10.1111/pbi.12170.

Tripura, Chaturvedula, Burla Sashidhar, and Appa Rao Podile. 2007. "Ethyl Methanesulfonate Mutagenesis-Enhanced Mineral Phosphate Solubilization by Groundnut-Associated Serratia Marcescens GPS-5." *Current Microbiology* 54 (2): 79–84. https://doi.org/10.1007/s00284-005-0334-1.

Tsai, Helen, Victor Missirian, Kathie J. Ngo, Robert K. Tran, Simon R. Chan, Venkatesan Sundaresan, and Luca Comai. 2013. "Production of a High-Efficiency TILLING Population through Polyploidization." *Plant Physiology* 161 (4): 1604–14. https://doi.org/10.1104/pp.112.213256.

Tyler, Brett M., Sucheta Tripathy, Xuemin Zhang, Paramvir Dehal, Rays H.Y. Jiang, Andrea Aerts, Felipe D. Arredondo, et al. 2006. "Phytophthora Genome Sequences Uncover Evolutionary Origins and Mechanisms of Pathogenesis." *Science* 313 (5791): 1261–66. https://doi.org/10.1126/science.1128796.

Upadhyaya, Narayana M., Jeffery G. Ellis, and Peter N. Dodds. 2014. "A Bacterial Type III Secretion-Based Delivery System for Functional Assays of Fungal Effectors in Cereals." *Methods in Molecular Biology* 1127: 277–90. https://doi.org/10.1007/978-1-62703-986-4_21.

Upadhyaya, Narayana M, Rohit Mago, Brian J Staskawicz, Michael A Ayliffe, Jeffrey G Ellis, and Peter N Dodds. 2014. "A Bacterial Type III Secretion Assay for Delivery of Fungal Effector Proteins into Wheat" 27 (3): 255–64. https://doi.org/10.1094/MPMI-07-13-0187-FI.

Vale, Franscisco, Xavier, Ribeiro, DO, J. E. Parlevliet, and Laercio Zambolim. 2001. "Concepts in Plant Disease Resistance." *Fitopatologia Brasileira* 26 (3): 577–89. https://doi.org/10.1590/s0100-41582001000300001.

Vetter, M. Madlen, Ilkka Kronholm, Fei He, Heidrun Häweker, Matthieu Reymond, Joy Bergelson, Silke Robatzek, and Juliette De Meaux. 2012. "Flagellin Perception Varies Quantitatively in Arabidopsis Thaliana and Its Relatives." *Molecular Biology and Evolution* 29 (6): 1655–67. https://doi.org/10.1093/molbev/mss011.

Visser, Botma, Marcel Meyer, Robert F. Park, Christopher A. Gilligan, Laura E. Burgin, Matthew C. Hort, David P. Hodson, and Zacharias A. Pretorius. 2019. "Microsatellite Analysis and Urediniospore Dispersal Simulations Support the Movement of *Puccinia*

Graminis f. Sp. *Tritici* from Southern Africa to Australia." *Phytopathology®* 109 (1): 133–44. https://doi.org/10.1094/PHYTO-04-18-0110-R.

Vleeshouwers, Vivianne G. A. A., and Richard P. Oliver. 2014. "Effectors as Tools in Disease Resistance Breeding Against Biotrophic, Hemibiotrophic, and Necrotrophic Plant Pathogens." *Molecular Plant-Microbe Interactions®* 27 (3): 196–206. https://doi.org/10.1094/MPMI-10-13-0313-IA.

Vleeshouwers, Vivianne G A A, Hendrik Rietman, Pavel Krenek, Nicolas Champouret, Carolyn Young, Sang Keun Oh, Miqia Wang, et al. 2008. "Effector Genomics Accelerates Discovery and Functional Profiling of Potato Disease Resistance and Phytophthora Infestans Avirulence Genes." Edited by Hany A. El-Shemy. *PLoS ONE* 3 (8): e2875. https://doi.org/10.1371/journal.pone.0002875.

Walker, Bruce J., Thomas Abeel, Terrance Shea, Margaret Priest, Amr Abouelliel, Sharadha Sakthikumar, Christina A. Cuomo, et al. 2014. "Pilon: An Integrated Tool for Comprehensive Microbial Variant Detection and Genome Assembly Improvement." Edited by Junwen Wang. *PLoS ONE* 9 (11): e112963. https://doi.org/10.1371/journal.pone.0112963.

Wan, Anmin, and Xianming Chen. 2014. "Virulence Characterization of Puccinia Striiformis f. Sp. Tritici Using a New Set of Yr Single-Gene Line Differentials in the United States in 2010." *Plant Disease* 98 (11): 1534–42. https://doi.org/10.1094/PDIS-01-14-0071-RE.

Wang, Bing, Yanfei Sun, Na Song, Mengxin Zhao, Rui Liu, Hao Feng, Xiaojie Wang, and Zhensheng Kang. 2017. "*Puccinia Striiformis* f. Sp. *Tritici* MicroRNA-like RNA 1 (*Pst* -MilR1), an Important Pathogenicity Factor of *Pst* , Impairs Wheat Resistance to *Pst* by Suppressing the Wheat Pathogenesis-Related 2 Gene." *New Phytologist* 215 (1): 338–50. https://doi.org/10.1111/nph.14577.

Wang, H., S. F. Hwang, F. Eudes, K. F. Chang, R. J. Howard, and G. D. Turnbull. 2006. "Trichothecenes and Aggressiveness of Fusarium Graminearum Causing Seedling Blight and Root Rot in Cereals." *Plant Pathology* 55 (2): 224–30. https://doi.org/10.1111/j.1365-3059.2006.01339.x.

Wang, Huan, Shenghao Zou, Yiwen Li, Fanyun Lin, and Dingzhong Tang. 2020. "An Ankyrin-Repeat and WRKY-Domain-Containing Immune Receptor Confers Stripe Rust Resistance in Wheat." *Nature Communications* 11 (1): 1–11. https://doi.org/10.1038/s41467-020-15139-6.

Wang, Hui, Abanti Chattopadhyay, Zhe Li, Bryce Daines, Yumei Li, Chunxu Gao, Richard Gibbs, Kun Zhang, and Rui Chen. 2010. “Rapid Identification of Heterozygous Mutations in Drosophila Melanogaster Using Genomic Capture Sequencing.” *Genome Research* 20 (7): 981–88. https://doi.org/10.1101/gr.102921.109.

Wang, Jizong, Meijuan Hu, Jia Wang, Jinfeng Qi, Zhifu Han, Guoxun Wang, Yijun Qi, Hong-Wei Wang, Jian-Min Zhou, and Jijie Chai. 2019a. “Reconstitution and Structure of a Plant NLR Resistosome Conferring Immunity.” *Science* 364 (6435): eaav5870. https://doi.org/10.1126/science.aav5870.

Wang, Jizong, Meijuan Hu, Jia Wang, Jinfeng Qi, Zhifu Han, Guoxun Wang, Yijun Qi, Hong Wei Wang, Jian Min Zhou, and Jijie Chai. 2019b. “Reconstitution and Structure of a Plant NLR Resistosome Conferring Immunity.” *Science* 364 (6435). https://doi.org/10.1126/science.aav5870.

Wang, Ming, Arne Weiberg, Feng Mao Lin, Bart P.H.J. Thomma, Hsien Da Huang, and Hailing Jin. 2016. “Bidirectional Cross-Kingdom RNAi and Fungal Uptake of External RNAs Confer Plant Protection.” *Nature Plants* 2 (10): 1–10. https://doi.org/10.1038/nplants.2016.151.

Wang, Xujing, Xin Zhang, Jiangtao Yang, Xiaojing Liu, Yaya Song, and Zhixing Wang. 2019. “Genetic Variation Assessment of Stacked-Trait Transgenic Maize via Conventional Breeding.” *BMC Plant Biology* 19 (1): 346. https://doi.org/10.1186/s12870-019-1956-y.

Wang, Yajun, Sudeep Subedi, Harmen de Vries, Pieter Doornenbal, Anton Vels, Goetz Hensel, Jochen Kumlehn, et al. 2019. “Orthologous Receptor Kinases Quantitatively Affect the Host Status of Barley to Leaf Rust Fungi.” *Nature Plants*. Palgrave Macmillan Ltd. https://doi.org/10.1038/s41477-019-0545-2.

Wanyera, R, J. K. Macharia, S M Kilonzo, and J W Kamundia. 2009. “Foliar Fungicides to Control Wheat Stem Rust, Race TTKS (Ug99), in Kenya.” *Plant Disease* 93 (9): 929–32. https://doi.org/10.1094/PDIS-93-9-0929.

Waterhouse, Robert M, Mathieu Seppey, Felipe A. Simao, Mosè Manni, Panagiotis Ioannidis, Guennadi Klioutchnikov, Evgenia V Kriventseva, and Evgeny M Zdobnov. 2018. “BUSCO Applications from Quality Assessments to Gene Prediction and Phylogenomics.” *Molecular Biology and Evolution* 35 (3): 543–48. https://doi.org/10.1093/molbev/msx319.

Watson, Amy, Sreya Ghosh, Matthew J. Williams, William S. Cuddy, James Simmonds, María

Dolores Rey, M. Asyraf Md Hatta, et al. 2018. “Speed Breeding Is a Powerful Tool to Accelerate Crop Research and Breeding.” *Nature Plants* 4 (1): 23–29. https://doi.org/10.1038/s41477-017-0083-8.

Webb, Craig A, Todd E Richter, Nicholas C Collins, Marie Nicolas, Harold N Trick, Tony Pryor, and Scot H Hulbert. 2002. “Genetic and Molecular Characterization of the Maize Rp3 Rust Resistance Locus.” *Genetics* 162 (1): 381–94. www.ncbi.nlm.nih.gov/blast/index.html.

Weiberg, Arne, Ming Wang, Feng-Mao Lin, Hongwei Zhao, Zhihong Zhang, Isgouhi Kaloshian, Hsien-Da Huang, and Hailing Jin. 2013. “Fungal Small RNAs Suppress Plant Immunity by Hijacking Host RNA Interference Pathways.” *Science* 342 (6154): 118–23. https://doi.org/10.1126/science.1239705.

Wersch, Solveig van, Lei Tian, Ryan Hoy, and Xin Li. 2020. “Plant NLRs: The Whistleblowers of Plant Immunity.” *Plant Communications* 1 (1): 100016. https://doi.org/10.1016/j.xplc.2019.100016.

Whisson, Stephen C., Petra C. Boevink, Lucy Moleleki, Anna O. Avrova, Juan G. Morales, Eleanor M. Gilroy, Miles R. Armstrong, et al. 2007. “A Translocation Signal for Delivery of Oomycete Effector Proteins into Host Plant Cells.” *Nature* 450 (7166): 115–18. https://doi.org/10.1038/nature06203.

Whisson, Stephen C, Petra C Boevink, Shumei Wang, and Paul Rj Birch. 2016. “The Cell Biology of Late Blight Disease.” *Current Opinion in Microbiology*. Elsevier. https://doi.org/10.1016/j.mib.2016.09.002.

Wiersma, Andrew T., Linda K. Brown, Elizabeth I. Brisco, Tiffany L. Liu, Kevin L. Childs, Jesse A. Poland, Sunish K. Sehgal, and Eric L. Olson. 2016. “Fine Mapping of the Stem Rust Resistance Gene SrTA10187.” *Theoretical and Applied Genetics* 129 (12): 2369–78. https://doi.org/10.1007/s00122-016-2776-1.

Williams, Simon J., Kee Hoon Sohn, Li Wan, Maud Bernoux, Panagiotis F. Sarris, Cecile Segonzac, Thomas Ve, et al. 2014. “Structural Basis for Assembly and Function of a Heterodimeric Plant Immune Receptor.” *Science* 344 (6181): 299–303. https://doi.org/10.1126/science.1247357.

Willow, Jonathan, Ana Silva, Eve Veromann, and Guy Smagghe. 2019. “Acute Effect of Low-Dose Thiacloprid Exposure Synergised by Tebuconazole in a Parasitoid Wasp.” Edited by James C. Nieh. *PLoS ONE* 14 (2): e0212456.

https://doi.org/10.1371/journal.pone.0212456.

Win, J., A. Chaparro-Garcia, K. Belhaj, D. G O Saunders, K. Yoshida, S. Dong, S. Schornack, et al. 2012. “Effector Biology of Plant-Associated Organisms: Concepts and Perspectives.” *Cold Spring Harbor Symposia on Quantitative Biology* 77: 235–47. https://doi.org/10.1101/sqb.2012.77.015933.

Winterberg, Britta, Lauren A. Du Fall, Xiaomin Song, Dana Pascovici, Natasha Care, Mark Molloy, Stephen Ohms, and Peter S. Solomon. 2014. “The Necrotrophic Effector Protein SnTox3 Re-Programs Metabolism and Elicits a Strong Defence Response in Susceptible Wheat Leaves.” *BMC Plant Biology* 14 (1): 1–15. https://doi.org/10.1186/s12870-014-0215-5.

Wit, P. J G M De, Rahim Mehrabi, Harrold A. Van Den Burg, and Ioannis Stergiopoulos. 2009. “Fungal Effector Proteins: Past, Present and Future: Review.” *Molecular Plant Pathology* 10 (6): 735–47. https://doi.org/10.1111/j.1364-3703.2009.00591.x.

Wit, P.J.G.M. De, M B Buurlage, and K E Hammond. 1986. “The Occurrence of Host-, Pathogen- and Interaction-Specific Proteins in the Apoplast of Cladosporium Fulvum (Syn. Fulvia Fulva) Infected Tomato Leaves.” *Physiological and Molecular Plant Pathology* 29 (2): 159–72. https://doi.org/10.1016/S0048-4059(86)80018-2.

Wit, Pierre J.G.M. de. 1981. “Physiological Studies on Cultivar-Specific Resistance of Tomato Plants To Cladosporium Fulvum.” *Physiological Plant Pathology* 16: 391–408.

Wit, Pierre J.G.M. De, Richard Laugé, Guy Honée, Matthieu H.A.J. Joosten, Paul Vossen, Miriam Kooman-Gersmann, Ralph Vogelsang, and Jacques J.M. Vervoort. 1997. “Molecular and Biochemical Basis of the Interaction between Tomato and Its Fungal Pathogen Cladosporium Fulvum.” In *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, 71:137–41. Antonie Van Leeuwenhoek. https://doi.org/10.1023/A:1000102509556.

Witek, Kamil, Florian Jupe, Agnieszka I Witek, David Baker, Matthew D Clark, and Jonathan D.G. Jones. 2016. “Accelerated Cloning of a Potato Late Blight-Resistance Gene Using RenSeq and SMRT Sequencing.” *Nature Biotechnology* 34 (6): 656–60. https://doi.org/10.1038/nbt.3540.

Wojtaszek, Przemysław. 1997. “Oxidative Burst: An Early Plant Response to Pathogen Infection.” *Biochemical Journal*. https://doi.org/10.1042/bj3220681.

Woldeab, Getaneh, Endale Hailu, and Netsanet Bacha. 2017. “Protocols for Race Analysis of

Wheat Stem Rust (Puccinia Graminis f. Sp. Tritici).” Ambo: Ethiopian Institute of Agricultural Research. www.globalrust.org/race-manual/Ambo.

Wolpert, T. J., V. Macko, W. Acklin, and D. Arigoni. 1988. “ Molecular Features Affecting the Biological Activity of the Host-Selective Toxins from Cochliobolus Victoriae .” *Plant Physiology* 88 (1): 37–41. https://doi.org/10.1104/pp.88.1.37.

Wouw, Angela P. Van de, Anton J. Cozijnsen, James K. Hane, Patrick C. Brunner, Bruce A. McDonald, Richard P. Oliver, and Barbara J. Howlett. 2010. “Evolution of Linked Avirulence Effectors in Leptosphaeria Maculans Is Affected by Genomic Environment and Exposure to Resistance Genes in Host Plants.” Edited by Anita Sil. *PLoS Pathogens* 6 (11): e1001180. https://doi.org/10.1371/journal.ppat.1001180.

Wróblewski, Tadeusz, Laurentiu Spiridon, Eliza Cristina Martin, Andrei Jose Petrescu, Keri Cavanaugh, Maria José Truco, Huaqin Xu, et al. 2018. “Genome-Wide Functional Analyses of Plant Coiled–Coil NLR-Type Pathogen Receptors Reveal Essential Roles of Their N-Terminal Domain in Oligomerization, Networking, and Immunity.” *PLoS Biology* 16 (12): e2005821. https://doi.org/10.1371/journal.pbio.2005821.

WSU Extension Resource. 2020. “Annual Stripe Rust Race Data Reports | Stripe Rust | Washington State University.” 2020. https://striperust.wsu.edu/races/data/.

Wu, Chih Hang, Ahmed Abd-El-Haliem, Tolga O. Bozkurt, Khaoula Belhaj, Ryohei Terauchi, Jack H. Vossen, and Sophien Kamoun. 2017. “NLR Network Mediates Immunity to Diverse Plant Pathogens.” *Proceedings of the National Academy of Sciences of the United States of America* 114 (30): 8113–18. https://doi.org/10.1073/pnas.1702041114.

Wulff, Brande B.H., Antje Hesse, Laurence Tomlinson-Buhot, David A. Jones, Marcos De La Peña, and Jonathan D.G. Jones. 2009. “The Major Specificity-Determining Amino Acids of the Tomato Cf-9 Disease Resistance Protein Are at Hypervariable Solvent-Exposed Positions in the Central Leucine-Rich Repeats.” *Molecular Plant-Microbe Interactions* 22 (10): 1203–13. https://doi.org/10.1094/MPMI-22-10-1203.

Wulff, Brande B H, and Matthew J Moscou. 2014. “Strategies for Transferring Resistance into Wheat: From Wide Crosses to GM Cassettes.” *Frontiers in Plant Science* 5 (December): 692. https://doi.org/10.3389/fpls.2014.00692.

Xia, Chongjing, Meinan Wang, Omar E Cornejo, Derick A Jiwan, Deven R See, and Xianming Chen. 2017. “Secretome Characterization and Correlation Analysis Reveal Putative

Pathogenicity Mechanisms and Identify Candidate Avirulence Genes in the Wheat Stripe Rust Fungus Puccinia Striiformis f. Sp. Tritici." *Frontiers in Microbiology* 8 (DEC): 2394. https://doi.org/10.3389/fmicb.2017.02394.

Xiong, Qin, Wenwu Ye, Duseok Choi, James Wong, Yongli Qiao, Kai Tao, Yuanchao Wang, and Wenbo Ma. 2014. "Phytophthora Suppressor of RNA Silencing 2 Is a Conserved RxLR Effector That Promotes Infection in Soybean and Arabidopsis Thaliana." *Molecular Plant-Microbe Interactions* 27 (12): 1379–89. https://doi.org/10.1094/MPMI-06-14-0190-R.

Xu, Ning, Xuming Luo, Wei Wu, Yingying Xing, Yingbo Liang, Yanzhi Liu, Huasong Zou, Hailei Wei, and Jun Liu. 2020. "A Plant Lectin Receptor-like Kinase Phosphorylates the Bacterial Effector AvrPtoB to Dampen Its Virulence in Arabidopsis." *Molecular Plant* 13 (10): 1499–1512. https://doi.org/10.1016/j.molp.2020.09.016.

Yang, Sihai, Xiaohui Zhang, Jia Xing Yue, Dacheng Tian, and Jian Qun Chen. 2008. "Recent Duplications Dominate NBS-Encoding Gene Expansion in Two Woody Species." *Molecular Genetics and Genomics* 280 (3): 187–98. https://doi.org/10.1007/s00438-008-0355-0.

Yin, Chuntao, James E. Jurgenson, and Scot H. Hulbert. 2011. "Development of a Host-Induced RNAi System in the Wheat Stripe Rust Fungus Puccinia Striiformis f. Sp. Tritici." *Molecular Plant-Microbe Interactions* 24 (5): 554–61. https://doi.org/10.1094/MPMI-10-10-0229.

Yin, Chuntao, Jeong Jin Park, David R. Gang, and Scot H. Hulbert. 2014. "Characterization of a Tryptophan 2-Monooxygenase Gene from Puccinia Graminis f. Sp. Tritici Involved in Auxin Biosynthesis and Rust Pathogenicity." *Molecular Plant-Microbe Interactions* 27 (3): 227–35. https://doi.org/10.1094/MPMI-09-13-0289-FI.

Yin, Chuntao, Sowmya R. Ramachandran, Ying Zhai, Chunya Bu, Hanu R. Pappu, and Scot H. Hulbert. 2019. "A Novel Fungal Effector from Puccinia Graminis Suppressing RNA Silencing and Plant Defense Responses." *New Phytologist* 222 (3): 1561–72. https://doi.org/10.1111/nph.15676.

Yirgou, Dagnatchew, and Ralph M. Caldwell. 1963. "Stomatal Penetration of Wheat Seedlings by Stem and Leaf Rust: Effect of Light and Carbon Dioxide." *Science* 141 (3577): 272–73. https://doi.org/10.1126/science.141.3577.272.

Yu, Guotai, Nicolas Champouret, Burkhard Steuernagel, Pablo D. Olivera, Jamie Simmons,

Cole Williams, Ryan Johnson, et al. 2017. “Discovery and Characterization of Two New Stem Rust Resistance Genes in Aegilops Sharonensis.” *Theoretical and Applied Genetics* 130 (6): 1207–22. https://doi.org/10.1007/s00122-017-2882-8.

Yu, Guotai, Oadi Matny, Brande B. H. Wulff, and Brian J. Steffenson. 2020. “The Stem Rust Resistance Gene Sr43 Encodes a Non-NLR Gene.” In *2020 BGRI Technical Workshop*. Norwich.

Yuan, Congying, Meinan Wang, Danniel Z. Skinner, Deven R. See, Chongjing Xia, Xinhong Guo, and Xianming Chen. 2018. “Inheritance of Virulence, Construction of a Linkage Map, and Mapping Dominant Virulence Genes in Puccinia Striiformis f. Sp. Tritici through Characterization of a Sexual Population with Genotyping-by-Sequencing.” *Phytopathology* 108 (1): 133–41. https://doi.org/10.1094/PHYTO-04-17-0139-R.

Yuan, Cuiling, Jingzheng Wu, Baiqiang Yan, Qunqun Hao, Chaozhong Zhang, Bo Lyu, Fei Ni, Allan Caplan, Jiajie Wu, and Daolin Fu. 2018. “Remapping of the Stripe Rust Resistance Gene Yr10 in Common Wheat.” *Theoretical and Applied Genetics* 131 (6): 1253–62. https://doi.org/10.1007/s00122-018-3075-9.

Zhang, Bo, Dawn Chi, Colin Hiebert, Tom Fetch, Brent McCallum, Allen Xue, Wenguang Cao, Ron Depauw, and George Fedak. 2019. “Pyramiding Stem Rust Resistance Genes to Race TTKSK (Ug99) in Wheat.” *Canadian Journal of Plant Pathology* 41 (3): 443–49. https://doi.org/10.1080/07060661.2019.1596983.

Zhang, Chaozhong, Lin Huang, Huifei Zhang, Qunqun Hao, Bo Lyu, Meinan Wang, Lynn Epstein, et al. 2019. “An Ancestral NB-LRR with Duplicated 3’UTRs Confers Stripe Rust Resistance in Wheat and Barley.” *Nature Communications 2019 10:1* 10 (1): 1–12. https://doi.org/10.1038/s41467-019-11872-9.

Zhang, Jianping, Peng Zhang, Peter Dodds, and Evans Lagudah. 2020. “How Target-Sequence Enrichment and Sequencing (TEnSeq) Pipelines Have Catalyzed Resistance Gene Cloning in the Wheat-Rust Pathosystem.” *Frontiers in Plant Science* 11 (May): 678. https://doi.org/10.3389/fpls.2020.00678.

Zhang, Meiping, Yang Zhang, Chantel F Scheuring, Cheng Cang Wu, Jennifer J Dong, and Hong Bin Zhang. 2012. “Preparation of Megabase-Sized DNA from a Variety of Organisms Using the Nuclei Method for Advanced Genomics Research.” *Nature Protocols* 7 (3): 467–78. https://doi.org/10.1038/nprot.2011.455.

Zhang, Ningwen W., Koen Pelgrom, Rients E. Niks, Richard G.F. Visser, and Marieke J.W.

Jeuken. 2009. “Three Combined Quantitative Trait Loci from Nonhost Lactuca Saligna Are Sufficient to Provide Complete Resistance of Lettuce against Bremia Lactucae.” *Molecular Plant-Microbe Interactions* 22 (9): 1160–68. https://doi.org/10.1094/MPMI-22-9-1160.

Zhang, Shijie, and Jin Rong Xu. 2014. “Effectors and Effector Delivery in Magnaporthe Oryzae.” Edited by Joseph Heitman. *PLoS Pathogens* 10 (1): e1003826. https://doi.org/10.1371/journal.ppat.1003826.

Zhang, Tao, Yun Long Zhao, Jian Hua Zhao, Sheng Wang, Yun Jin, Zhong Qi Chen, Yuan Yuan Fang, Chen Lei Hua, Shou Wei Ding, and Hui Shan Guo. 2016. “Cotton Plants Export MicroRNAs to Inhibit Virulence Gene Expression in a Fungal Pathogen.” *Nature Plants* 2 (10): 1–6. https://doi.org/10.1038/nplants.2016.153.

Zhang, Wen Jing, Carsten Pedersen, Mark Kwaaitaal, Per L. Gregersen, Sara M. Mørch, Susanne Hanisch, Astrid Kristensen, Anja T. Fuglsang, David B. Collinge, and Hans Thordal-Christensen. 2012. “Interaction of Barley Powdery Mildew Effector Candidate CSEP0055 with the Defence Protein PR17c.” *Molecular Plant Pathology* 13 (9): 1110–19. https://doi.org/10.1111/j.1364-3703.2012.00820.x.

Zhang, Wenjun, Shisheng Chen, Zewdie Abate, Jayaveeramuthu Nirmala, Matthew N. Rouse, and Jorge Dubcovsky. 2017. “Identification and Characterization of Sr13, a Tetraploid Wheat Gene That Confers Resistance to the Ug99 Stem Rust Race Group.” *Proceedings of the National Academy of Sciences of the United States of America* 114 (45): E9483–92. https://doi.org/10.1073/pnas.1706277114.

Zhang, Xiaoxiao, Nadya Farah, Laura Rolston, Daniel J. Ericsson, Maud Bernoux, Thomas Ve, Katerina Bendak, et al. 2006. “Crystal Structure of the Melampsora Lini Effector AvrP Reveals Insights into a Possible Nuclear Function and Recognition by the Flax Disease Resistance Protein P.” *Molecular Plant Pathology*, August. https://doi.org/10.1111/mpp.12597.

Zhang, Zhi Min, Xu Zhang, Zi Ren Zhou, Hong Yu Hu, Maili Liu, Bo Zhou, and Jiahai Zhou. 2013. “Solution Structure of the Magnaporthe Oryzae Avirulence Protein AvrPiz-T.” *Journal of Biomolecular NMR* 55 (2): 219–23. https://doi.org/10.1007/s10858-012-9695-5.

Zhao, Mengxin, Jianfeng Wang, Sen Ji, Zengju Chen, Jinghua Xu, Chunlei Tang, Shuntao Chen, Zhensheng Kang, and Xiaojie Wang. 2018. “Candidate Effector Pst_8713 Impairs

the Plant Immunity and Contributes to Virulence of Puccinia Striiformis f. Sp. Tritici." *Frontiers in Plant Science* 9 (September). https://doi.org/10.3389/fpls.2018.01294.

Zhao, Shuqing, Xiaofeng Shang, Weishuai Bi, Xiumei Yu, Daqun Liu, Zhensheng Kang, Xiaojie Wang, and Xiaodong Wang. 2020. "Genome-Wide Identification of Effector Candidates With Conserved Motifs From the Wheat Leaf Rust Fungus Puccinia Triticina." *Frontiers in Microbiology* 11 (June): 1188. https://doi.org/10.3389/fmicb.2020.01188.

Zhong, Ziming, Thierry C. Marcel, Fanny E. Hartmann, Xin Ma, Clémence Plissonneau, Marcello Zala, Aurélie Ducasse, et al. 2017. "A Small Secreted Protein in Zymoseptoria Tritici Is Responsible for Avirulence on Wheat Cultivars Carrying the Stb6 Resistance Gene." *New Phytologist* 214 (2): 619–31. https://doi.org/10.1111/nph.14434.

Zhu, Xiaoguo, Jia Guo, Fuxin He, Yang Zhang, Chenglong Tan, Qian Yang, Chuanming Huang, Zhensheng Kang, and Jun Guo. 2018. "Silencing PsKPP4, a MAP Kinase Kinase Kinase Gene, Reduces Pathogenicity of the Stripe Rust Fungus." *Molecular Plant Pathology* 19 (12): 2590–2602. https://doi.org/10.1111/mpp.12731.

Zhu, Xiaoguo, Min Jiao, Jia Guo, Peng Liu, Chenglong Tan, Qian Yang, Yang Zhang, Ralf Thomas Voegele, Zhensheng Kang, and Jun Guo. 2018. "A Novel MADS-Box Transcription Factor *PstMCM1-1* Is Responsible for Full Virulence of *Puccinia Striiformis* f. Sp. *Tritici*." *Environmental Microbiology* 20 (4): 1452–63. https://doi.org/10.1111/1462-2920.14054.

Zhu, Xiaoguo, Wei Liu, Xiuling Chu, Qixiong Sun, Chenglong Tan, Qian Yang, Min Jiao, Jun Guo, and Zhensheng Kang. 2018. "The Transcription Factor PstSTE12 Is Required for Virulence of Puccinia Striiformis f. Sp. Tritici." *Molecular Plant Pathology* 19 (4): 961–74. https://doi.org/10.1111/mpp.12582.

Zhu, Xiaoguo, Tuo Qi, Qian Yang, Fuxin He, Chenglong Tan, Wei Ma, Ralf Thomas Voegele, Zhensheng Kang, and Jun Guo. 2017. "Host-Induced Gene Silencing of the MAPKK Gene PsFUZ7 Confers Stable Resistance to Wheat Stripe Rust." *Plant Physiology* 175 (4): 1853–63. https://doi.org/10.1104/pp.17.01223.

Zipfel, Cyril. 2008. "Pattern-Recognition Receptors in Plant Innate Immunity." *Current Opinion in Immunology*. https://doi.org/10.1016/j.coi.2007.11.003.

———. 2009. "Early Molecular Events in PAMP-Triggered Immunity." *Current Opinion in Plant Biology*. https://doi.org/10.1016/j.pbi.2009.06.003.

Zipfel, Cyril, Gernot Kunze, Delphine Chinchilla, Anne Caniard, Jonathan D G Jones, Thomas

Boller, and Georg Felix. 2006. “Perception of the Bacterial PAMP EF-Tu by the Receptor EFR Restricts Agrobacterium-Mediated Transformation.” *Cell* 125 (4): 749–60. https://doi.org/10.1016/j.cell.2006.03.037.

Zwiers, L. H., I. Stergiopoulos, M. M C Gielkens, S. D. Goodall, and M. A. De Waard. 2003. “ABC Transporters of the Wheat Pathogen Mycosphaerella Graminicola Function as Protectants against Biotic and Xenobiotic Toxic Compounds.” *Molecular Genetics and Genomics* 269 (4): 499–507. https://doi.org/10.1007/s00438-003-0855-x.

7. Chapter 7. Thesis Annexe

7.1 Annex 1. Wheat-alien introgression lines and Aegilops tauschii accessions carrying single Sr genes that were used in the experiments

Identifier	Description	Line	Source	Reference
Sr22	*Triticum. aestivum* cv. Schomburgk carrying *Sr22* derived from *T. boeoticum* accession G-21	W3534	Evans Lagudah, CSIRO, Canberra, Australia	(Paull *et al.*, 1994)
Sr25	*T. aestivum* carrying *Sr25* derived from *Thinopyrum ponticum*	Sr25L-2A-1	Michael Pumphrey, Washington State University, USA	Michael Pumphrey, personal communication
Sr33	*T. aestivum* carrying *Sr33* derived from *Aegilops. tauschii*	CSID5405	Evans Lagudah, CSIRO, Canberra, Australia	(Jones *et al.*, 1991)
Sr40	*T. aestivum* cv. Westinonia carrying *Sr40* derived from *T. timopheevii*	2B-2G#2 translocation (Sr40-1 x W(2)) (Westonia backcrosses) (+Sr40), source 1410/12	Ian Dundas, University of Adelaide, Australia	Ian Dundas, personal communication
Sr43	*T. aestivum* carrying Sr43 derived from *Th. ponticum*	RWG34	Steven Xu, USDA, Fargo, USA	(Niu *et al.*, 2014)
Sr44	*T. aestivum* cv. Angus carrying *Sr44* derived from *Th. intermedium*	IK1019 x Angas, source 1350/13	Ian Dundas, University of Adelaide, Australia	(Khan, 1996)
Sr45	*T. aestivum* carrying *Sr45* derived from *Ae. tauschii*	CSID5406	Evans Lagudah, CSIRO, Canberra, Australia	(Periyannan *et al.*, 2014)
Sr45	*Ae. tauschii* line TOWWC191; contains *Sr45* but not *Sr33*, *Sr46* or *SrTA1662*	TOWWC0191	Germplasm Resources Unit, John Innes Centre, UK	(Arora *et al.*, 2019)
Sr46	*Ae. tauschii* line TOWWC151; contains *Sr46*, but not *Sr33*, *Sr45* or *SrTA1662*	TOWWC0151	Germplasm Resources Unit, John Innes Centre, UK	(Arora *et al.*, 2019)
Sr51	*T. aestivum* with *Sr51* derived from *Ae. searsii*		Michael Pumphrey, Washington State Unviersity, USA	(Liu, Jin, *et al.*, 2011)

Identifier	Description	Line	Source	Reference
Sr53	*T. aestivum* with *Sr53* derived from *Ae. geniculata*	TA5630	Michael Pumphrey, Washington State Unviersity, USA	(Liu, Rouse, *et al.*, 2011)
SrTA1662	*Ae. tauschii* line TOWWC017; contains *SrTA1662* but not *Sr33*, *Sr45* or *Sr46*	TOWWC0017	Germplasm Resources Unit, John Innes Centre, UK	(Arora *et al.*, 2019)
Sr1644-1Sh	*T. aestivum* accession with *Sr1664-1Sh* introgressed from *Ae. sharonensis*		Eitan Millet, Institute for Cereal Crops Improvement, Tel Aviv, Israel	(Millet *et al.*, 2017)
Sr2020	*Ae. sharonensis* accession 2020			(Yu *et al.*, 2017)
Recurrent parent for *Sr22*	*T. aestivum* cv. Schomburgk		Evans Lagudah, CSIRO, Canberra, Australia	
Recurrent parent for *Sr25*	*T. aestivum* cv. Louise		Michael Pumphrey, Washington State Unviersity, USA	
Recurrent parent for *Sr40*	*T. aestivum* cv. Westonia	Source 629/08	Ian Dundas, University of Adelaide, Australia	
Recurrent parent for *Sr44*	*T. aestivum* cv. Angus		Ian Dundas, University of Adelaide, Australia	
Recurrent parent for *Sr1644-1Sh*	*T. aestivum* cv. Zahir		Eitan Millet, Institute for Cereal Crops Improvement, Tel Aviv, Israel	
Recurrent parent for *Sr33*, *Sr43*, *Sr45, Sr51*, *Sr53*	*T. aestivum* cv. Chinese Spring		Michael Bevan, John Innes Centre	
Susceptible control	*T. aestivum* cv. Vuka	Toering/Merlin /2/Carsten 8	Minas Seed Co-operative Ltd, Canning, Nova Scotia	
Resistant control *Sr31*	*T. aestivum* carrying *Sr31* derived from *Secale cereale*	Kavkaz/Federation4	Tom Fetch, Cereal Research Centre, Winnipeg, Canada	

7.2 Annexe 2. EMS experiment pustule count (A) and Analysis of Variance (ANOVA) of pustule count in EMS experiment 1 (B)

A

Pot	0 M	0.015 M	0.025 M	0.05 M	0.075 M
1	3	1	4	0	0
2	6	1	3	4	0
3	1	2	3	1	0
4	0	0	1	1	1
5	3	5	3	1	1
6	1	0	3	0	1
7	1	0	3	4	0
Treatment total	**16**	**8**	**20**	**11**	**3**
Mutant pustule total	**52**				

B

ANOVA of pustule count from EMS experiment 1					
	Sum of Squares	Degrees of freedom	Mean Square	F	Significance
Between Groups	25.314	4	6.329	3.034	0.063
Within Groups	62.571	30	2.086		
Total	87.886	34			

7.3 Annexe 3. Number of *Pgt* pustules from EMS experiment 2.

Pot number	Plant	0 M	0.015 M	0.025 M	0.05 M	0.075 M
Sample pot 1	1	27	51	40	69	6
	2	25	25	24	37	3
	3	49	60	40	19	10
	4	37	33	52	26	7
	5	15	40	24	52	7
	6	31	42	29	61	11
	7	20	43	35	30	11
	Pot total	**204**	**294**	**244**	**294**	**55**
Sample pot 2	8	35	41	29	51	7
	9	52	44	18	34	2
	10	105	49	19	94	5
	11	56	60	10	49	6
	12	55	38	25	42	4
	13	84	62	44	13	8
	14	64	37	15	9	3
	Pot total	**451**	**331**	**160**	**292**	**35**
Sample pot 3	15	93	42	27	29	2
	16	62	55	16	20	10
	17	62	39	25	22	7
	18	82	30	40	28	0
	19	40	33	16	33	2
	20	37	30	19	40	0
	21	69	33	14	35	3
	22	81	25	27	31	4
	Pot total	**526**	**287**	**184**	**238**	**28**
Sample pot 4	23	54	0	0	63	8
	24	88	0	0	21	6
	25	79	0	0	29	10
	26	79	0	0	23	6
	27	38	0	0	0	8
	28	100	0	0	0	12
	29	88	0	0	0	4
	30	84	0	0	0	0
	Pot total	**610**	**0**	**0**	**136**	**54**
Total for 4 pots		**1791**	**912**	**588**	**960**	**172**
Extrapolation to experiment total (16 pots)		**7164**	**3648**	**2352**	**3840**	**688**

7.4 Annexe 4. EMS experiment 3 Pustule count

Pot number	Plant	0 M	0.015 M	0.025 M	0.05 M	0.075 M
Sample pot 1	1	21	6	11	6	3
	2	26	13	3	0	1
	3	31	10	3	2	1
	4	14	10	6	7	2
	5	23	11	3	2	0
	6	29	20	4	0	1
	7	21	17	1	1	0
	8	27	0	5	2	1
	Pot total	**192**	**87**	**36**	**20**	**9**
Sample pot 2	9	25	11	7	4	1
	10	48	5	11	3	2
	11	31	13	11	0	2
	12	42	5	14	0	8
	13	40	8	5	1	3
	14	49	14	10	4	2
	15	39	12	7	2	1
	16	48	6	3	1	1
	Pot total	**322**	**74**	**68**	**15**	**20**
Sample pot 3	17	46	6	7	0	1
	18	35	10	10	0	1
	19	39	12	12	1	2
	20	21	15	10	2	3
	21	37	8	3	5	0
	22	22	3	13	0	2
	23	44	22	9	0	1
	24	21	8	4	2	1
	Pot total	**265**	**84**	**68**	**10**	**11**
Sample pot 4	25	64	14	1	3	0
	26	39	3	3	1	1
	27	44	8	1	2	1
	28	62	3	7	2	3
	29	56	8	2	6	4
	30	33	4	3	3	0
	31	25	10	8	4	0
	32	27	4	3	3	0
	Pot total	**350**	**54**	**28**	**24**	**9**
Total for 4 pots		**1129**	**299**	**200**	**69**	**49**
Extrapolation to experiment total (16 pots)		**4516**	**1196**	**800**	**276**	**156**

7.5 Annexe 5. ANOVA and LSD for EMS experiment 2

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Block	Between Groups	.000	4	.000	.000	1.000
	Within Groups	25.000	15	1.667		
	Total	25.000	19			
Pustule Count	Between Treatments	208850.800	4	52212.700	10.669	.000
	Within Treatments	73407.750	15	4893.850		
	Total	282258.550	19			

Multiple Comparisons

Dependent Variable: Normalised pustule count

	(I) EMS Level	(J) EMS Level	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LSD	A 0 M	B	92.00	49.466	.083	-13.44	197.44
		C	186.00*	49.466	.002	80.56	291.44
		D	96.50	49.466	.070	-8.94	201.94
		E	302.50*	49.466	.000	197.06	407.94
	B 0.015 M	A	-92.00	49.466	.083	-197.44	13.44
		C	94.00	49.466	.077	-11.44	199.44
		D	4.50	49.466	.929	-100.94	109.94
		E	210.50*	49.466	.001	105.06	315.94
	C 0.025 M	A	-186.00*	49.466	.002	-291.44	-80.56
		B	-94.00	49.466	.077	-199.44	11.44
		D	-89.50	49.466	.090	-194.94	15.94
		E	116.50*	49.466	.033	11.06	221.94
	D 0.05 M	A	-96.50	49.466	.070	-201.94	8.94
		B	-4.50	49.466	.929	-109.94	100.94
		C	89.50	49.466	.090	-15.94	194.94
		E	206.00*	49.466	.001	100.56	311.44
	E 0.075 M	A	-302.50*	49.466	.000	-407.94	-197.06
		B	-210.50*	49.466	.001	-315.94	-105.06
		C	-116.50*	49.466	.033	-221.94	-11.06
		D	-206.00*	49.466	.001	-311.44	-100.56

Based on observed means.

The error term is Mean Square (Error) = 4893.850.

7.6 Annexe 6. ANOVA and LSD for EMS experiment 3

ANOVA

Pustule count					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	200375.200	4	50093.800	44.724	.000
Within Groups	16801.000	15	1120.067		
Total	217176.200	19			

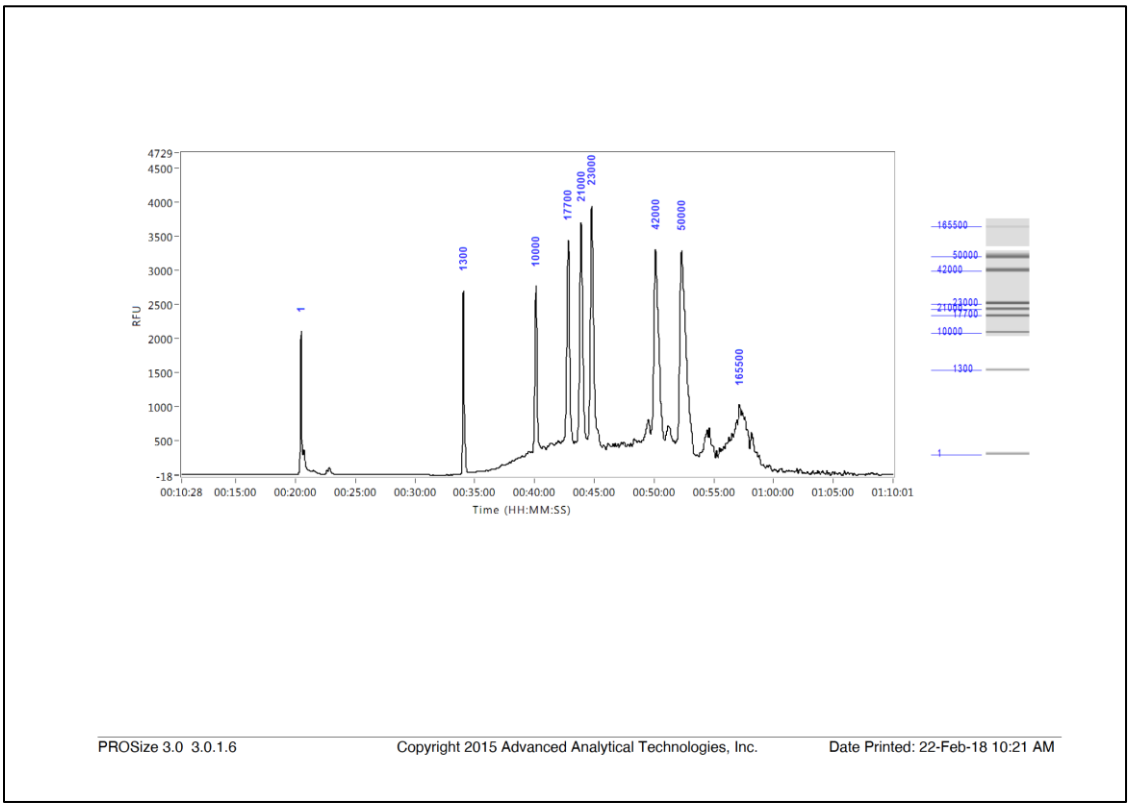
Multiple Comparisons

Dependent Variable: Pustule Count

						95% Confidence Interval	
	(I) EMS Level	(J) EMS Level	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
LSD	1.00 0 M	2.00	207.50000*	23.66502	.000	157.0592	257.9408
		3.00	232.25000*	23.66502	.000	181.8092	282.6908
		4.00	265.00000*	23.66502	.000	214.5592	315.4408
		5.00	270.00000*	23.66502	.000	219.5592	320.4408
	2.00 0.015 M	1.00	-207.50000*	23.66502	.000	-257.9408	-157.0592
		3.00	24.75000	23.66502	.312	-25.6908	75.1908
		4.00	57.50000*	23.66502	.028	7.0592	107.9408
		5.00	62.50000*	23.66502	.019	12.0592	112.9408
	3.00 0.025 M	1.00	-232.25000*	23.66502	.000	-282.6908	-181.8092
		2.00	-24.75000	23.66502	.312	-75.1908	25.6908
		4.00	32.75000	23.66502	.187	-17.6908	83.1908
		5.00	37.75000	23.66502	.132	-12.6908	88.1908
	4.00 0.05 M	1.00	-265.00000*	23.66502	.000	-315.4408	-214.5592
		2.00	-57.50000*	23.66502	.028	-107.9408	-7.0592
		3.00	-32.75000	23.66502	.187	-83.1908	17.6908
		5.00	5.00000	23.66502	.836	-45.4408	55.4408
	5.00 0.075 M	1.00	-270.00000*	23.66502	.000	-320.4408	-219.5592
		2.00	-62.50000*	23.66502	.019	-112.9408	-12.0592
		3.00	-37.75000	23.66502	.132	-88.1908	12.6908
		4.00	-5.00000	23.66502	.836	-55.4408	45.4408

*. The mean difference is significant at the 0.05 level.

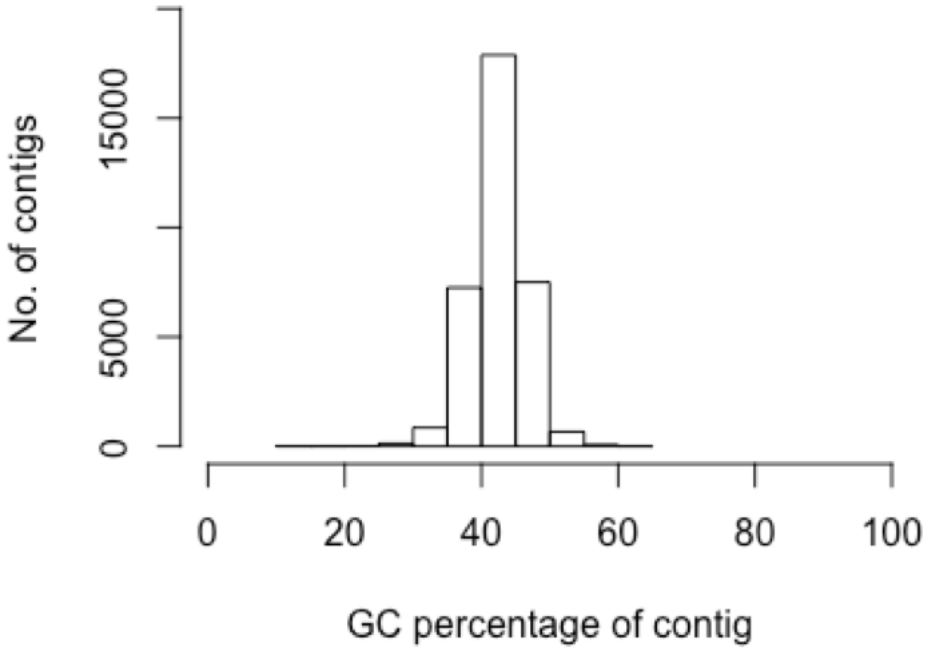
7.7 Annexe 7. Q-Card report for agarose plug DNA extraction



7.8 Annexe 8. Correlation of SNV densities with EMS concentration

Dependent Variable: High confidence EMS SNV count					
Source	**Type III Sum of Squares**	**df**	**MS**	**F**	**Significance**
Corrected Model	697341.5a	4	174335.375	16.888	0.021
Intercept	1,820,885.786	1	1820885.786	176.394	0.001
EMS Level	69,7341.5	4	174335.375	16.888	0.021
Error	3,0968.5	3	10322.833		
Total	3,391,742	8			
Corrected Total	728,310	7			
aR Squared = 0.957 (Adjusted R Squared = 0.901)					

7.9 Annexe 9. Distribution of G:C content amongst contigs



7.10 Annexe 10. Infection types of gain-of-virulence mutants identified on *Sr43*, *Sr44* and *Sr45*

Identifier	***S43* IT**	**Identifier**	***Sr44* IT**	**Identifier**	***Sr45* IT**
Wildtype	1+	Wildtype	;	Wildtype	1
AvrSr43 M-1	3	AvrSr44 M-1	1+	AvrSr45 M-1	3
AvrSr43 M-3	4	AvrSr44 M-2	1	AvrSr45 M-2	3
AvrSr43 M-4	3+	AvrSr44 M-3	1+	AvrSr45 M-3	2+
AvrSr43 M-5	3	AvrSr44 M-4	1+	AvrSr45 M-4	4
AvrSr43 M-6	4			AvrSr45 M-5	4
AvrSr43 M-7	3			AvrSr45 M-6	4
AvrSr43 M-8	4			AvrSr45 M-7	2+
AvrSr43 M-9	4			AvrSr45 M-8	3
AvrSr43 M-10	3			AvrSr45 M-9	2+
AvrSr43 M-11	4			AvrSr45 M-10	4
AvrSr43 M-12	4			AvrSr45 M-11	2+
AvrSr43 M-13	4			AvrSr45 M-12	3
				AvrSr45 M-13	3
				AvrSr45 M-14	3

7.11 Annexe 11. Infection types of wild type and mutant *Pgt* UK-01 on differential lines

Set	Cultivar	Gene	Inoculations with *Pgt* UK-01									Race
			Infection type									
1	ISr5-Ra CI 14159	*Sr5*	4	4	4	4	4	4	4	4	4	T
	*T monococcum/8*LMPG-6 DK13*	*Sr21*	3	4	4	3	4	3	4	4	4	
	Vernstein PI 442914	*Sr9e*	4	4	4	4	4	4	4	4	4	
	ISr7b-Ra CI 14165	*Sr7b*	3	3	3	3	3	4	3	3	3	
2	Yalta PI 155433	*Sr11*	1	1	1	1	2	2	2	2	2	K
	ISr6-Ra CI 14163	*Sr6*	4	4	4	4	4	4	4	4	4	
	Mentana W1124 PI 221154	*Sr8a*	4	4	4	4	4	4	4	4	4	
	Acme CI 5284	*Sr9g*	4	4	4	4	4	4	4	4	4	
3	W2691SrTt-1 CI 17385	*Sr36*	4	4	4	4	4	4	4	4	4	T
	Prelude*4/2/Marquis*6/Kenya 117A	*Sr9b*	4	4	4	4	4	4	4	4	4	
	Festiguay W2706 PI 330957	*Sr30*	4	4	4	4	4	4	4	4	4	
	Prelude/8*Marquis*2/2/Esp 518/9	*Sr17*	4	4	4	4	4	4	4	4	4	
4	ISr9a-Ra CI 14169	*Sr9a*	4	4	4	4	4	4	4	4	4	T
	ISr9d-Ra CI 14177	*Sr9d*	4	4	4	3	3	4	4	3+	4	
	W2691Sr10 CI 17388	*Sr10*	3+	3	4	4	3	3	4	4	4	
	CnsSrTmp	*SrTmp*	4	4	4	4	4	4	4	4	4	
5	LcSr24Ag	*Sr24*	2-	1+	1+	1+	2-	2-	2	2-	2-	F
	Kavkaz/Federation4	*Sr31*	;	1-	1-	;1-	;1-	;1-	1-	;	1-	
	VPM1	*Sr38*	4	4	4	4	4	3+	4	4	3	
	McNair 701 (CI 15288)	*SrMcN*	3+	4	4	4	4	4	4	4	4	

7.12 Annexe 12. North American Rust Nomenclature Code Sheet

	Four gene differential sets				
	Sr5	*Sr21*	*Sr9e*	*Sr7b*	Set 1
	Sr11	*Sr6*	*Sr8a*	*Sr9g*	Set 2
	Sr36	*Sr9b*	*Sr30*	*Sr17*	Set 3
	Sr9a	*Sr9d*	*Sr10*	*SrTmp*	Set 4
Pgt letter	*Sr24*	*Sr31*	*Sr38*	*SrMcN*	Set 5
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	

H = High Infection Type (3-4 on standard evaluation scale)
L = Low Infection Type (0-2 on standard evaluation scale)

Example: Race TTKSK (original Ug99)

Set 1: H H H H = T (No genes in set are effective)
Set 2: H H H H = T (No genes in set are effective)
Set 3: L H H H = K (Only gene Sr36 is effective)
Set 4: H H H L = S (Only gene SrTmp is effective)
Set 5: L H H H = K (Only gene Sr24 is effective)

7.13 Annexe 13. Chitin fluorescence readings for mutant E1

	24h	48h	6DPI	16DPI	24h	48h	6DPI	16DPI	24h	48h	6DPI	16DPI
	1	2	3	4	1	2	3	4	1	2	3	4
A	WTxSr43	WTxSr43	WTxSr43	WTxSr43	WTxCS	WTxCS	WTxCS	WTxCS	E1xSr43	E1xSr43	E1xSr43	E1xSr43
B	WTxSr43	WTxSr43	WTxSr43	WTxSr43	WTxCS	WTxCS	WTxCS	WTxCS	E1xSr43	E1xSr43	E1xSr43	E1xSr43
C	WTxSr43	WTxSr43	WTxSr43	WTxSr43	WTxCS	WTxCS	WTxCS	WTxCS	E1xSr43	E1xSr43	E1xSr43	E1xSr43
D	WTxSr43	WTxSr43	WTxSr43	WTxSr43	WTxCS	WTxCS	WTxCS	WTxCS	E1xSr43	E1xSr43	E1xSr43	E1xSr43
E	WTxSr43	WTxSr43	WTxSr43	WTxSr43	WTxCS	WTxCS	WTxCS	WTxCS	E1xSr43	E1xSr43	E1xSr43	E1xSr43
F	WTxSr43	WTxSr43	WTxSr43	WTxSr43	WTxCS	WTxCS	WTxCS	WTxCS	E1xSr43	E1xSr43	E1xSr43	E1xSr43
G	WTxSr43	WTxSr43	WTxSr43	WTxSr43	WTxCS	WTxCS	WTxCS	WTxCS	E1xSr43	E1xSr43	E1xSr43	E1xSr43
H	Tris HCL	Tris HCL	Tris HCL	Tris HCL	Tris HCL	Tris HCL	Tris HCL	Tris HCL	Tris HCL	Tris HCL	Tris HCL	Tris HCL
	1	2	3	4	1	2	3	4	1	2	3	4
A	48.35	23.71	23.14	52.58	45.01	31.84	29.09	295.6	48.99	52.29	46.73	220.3
B	24.93	20.32	43.63	47.31	37.35	34.98	34.29	201.1	29.8	49.95	52.9	167.1
C	10.18	29.91	64.23	43.41	21.9	28.52	115.3	46.93	26.39	44.99	42.95	110.2
D	15.9	64.83	31.69	50.44	25.01	29.42	34.36	124.6	25.82	23.32	32.55	86.02
E	23.31	35.36	25.99	60.14	21.99	36.55	27.27	186.2	21.1	31.76	34.62	140
F	71.62	72.04	63.46	47.91	37.62	30.25	34.02	155.8	55.63	53.48	47.2	141.8
G	14.53	47.35	41.8	38.27	90.45	44.01	31.14	31.53	31.63	49.98	39.62	147
H	0.00675	0.003455	0.005792	0.005829	0.007539	0.007135	0.006982	0.004798	0.007834	0.00531	0.007829	0.007437
Total	**208.82**	**293.52**	**293.94**	**340.06**	**279.33**	**235.57**	**305.47**	**1041.76**	**239.36**	**305.77**	**296.57**	**1012.42**

7.14 Annexe 14 Chitin fluorescence readings for mutant E7-1

	24h	48h	6DPI	16DPI	24h	48h	6DPI	16DPI	24h	48h	6DPI	16DPI
	1	2	3	4	1	2	3	4	1	2	3	4
A	WTxSr43	WTxSr43	WTxSr43	WTxSr43	WTxCS	WTxCS	WTxCS	WTxCS	E7-1xSr43	E7-1xSr43	E7-1xSr43	E7-1xSr43
B	WTxSr43	WTxSr43	WTxSr43	WTxSr43	WTxCS	WTxCS	WTxCS	WTxCS	E7-1xSr43	E7-1xSr43	E7-1xSr43	E7-1xSr43
C	WTxSr43	WTxSr43	WTxSr43	WTxSr43	WTxCS	WTxCS	WTxCS	WTxCS	E7-1xSr43	E7-1xSr43	E7-1xSr43	E7-1xSr43
D	WTxSr43	WTxSr43	WTxSr43	WTxSr43	WTxCS	WTxCS	WTxCS	WTxCS	E7-1xSr43	E7-1xSr43	E7-1xSr43	E7-1xSr43
E	WTxSr43	WTxSr43	WTxSr43	WTxSr43	WTxCS	WTxCS	WTxCS	WTxCS	E7-1xSr43	E7-1xSr43	E7-1xSr43	E7-1xSr43
F	WTxSr43	WTxSr43	WTxSr43	WTxSr43	WTxCS	WTxCS	WTxCS	WTxCS	E7-1xSr43	E7-1xSr43	E7-1xSr43	E7-1xSr43
G	WTxSr43	WTxSr43	WTxSr43	WTxSr43	WTxCS	WTxCS	WTxCS	WTxCS	E7-1xSr43	E7-1xSr43	E7-1xSr43	E7-1xSr43
H	Tris HCL	Tris HCL	Tris HCL	Tris HCL	Tris HCL	Tris HCL	Tris HCL	Tris HCL	Tris HCL	Tris HCL	Tris HCL	Tris HCL
	1	2	3	4	1	2	3	4	1	2	3	4
A	118.12	123.10	138.10	212.40	32.70	133.80	241.00	306.80	87.58	261.62	189.60	437.21
B	119.23	132.80	110.70	204.00	122.30	96.20	255.50	254.60	71.17	179.40	399.50	336.90
C	86.00	181.70	195.40	280.80	101.10	110.65	282.00	514.00	91.74	127.26	195.30	237.61
D	247.10	143.60	162.90	196.30	123.70	99.07	251.40	333.50	85.75	29.99	202.50	327.31
E	115.21	67.50	172.00	273.40	110.00	209.71	224.70	391.60	130.89	43.42	203.60	354.90
F	83.10	153.20	180.10	71.60	126.80	75.48	247.10	294.60	151.47	26.31	244.90	312.70
G	61.10	144.30	144.20	117.10	133.00	309.29	226.60	379.40	93.69	358.71	224.10	352.30
H	0.00147	0.0078	0.0011	0.00731	0.0027	0.0059	0.0033	0.00921	0.0063	0.001	0.00413	0.00971
Total	**829.86**	**946.21**	**1103.40**	**1355.61**	**749.60**	**1034.21**	**1728.30**	**2474.51**	**712.30**	**1026.71**	**1659.50**	**2358.94**

7.15 Annexe 15. Chitin fluorescence readings for E1 wildtype *Pgt* UK-01 and its mutant E1 virulent on *Sr43*.

ANOVA

Fluorescence

	Sum of Squares	df	Mean Square	F	Sig.
Between treatments	228988.130	2	114494.065	9.111	.000
Within treatments	1017937.968	81	12567.135		
Total	1246926.098	83			

Fisher's Least Significant Difference

Dependent Variable: Fluorescence							
			Mean			95% Confidence Interval	
	(I) Treatment	(J) Treatment	Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
LSD	(1) Wildtype x Sr43	2	-118.38007*	29.96085	.000	-177.9928	-58.7674
		3	-101.10471*	29.96085	.001	-160.7174	-41.4920
	(2) E1 x Sr43	1	118.38007*	29.96085	.000	58.7674	177.9928
		3	17.27536	29.96085	.566	-42.3373	76.8881
	(3) WT x Chinese Spr.	1	101.10471*	29.96085	.001	41.4920	160.7174
		2	-17.27536	29.96085	.566	-76.8881	42.3373

*. The mean difference is significant at the 0.05 level.

7.16 Annexe 16. Chitin fluorescence readings for wildtype *Pgt* UK-01 and its mutant E7-1 virulent on *Sr43*.

ANOVA

Fluorescence

	Sum of Squares	df	Mean Square	F	Sig.
Between treatments	64738.353	2	32369.177	3.174	.047
Within treatments	826142.551	81	10199.291		
Total	890880.904	83			

Means Comparisons (LSD)

Dependent Variable: Fluorescence							
			Mean			95% Confidence Interval	
	(I) Treatment	(J) Treatment	Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
LSD	Wildtype x Sr43	2.00	-62.55500*	26.99112	.023	-116.2589	-8.8511
		3.00	-54.37036*	26.99112	.047	-108.0742	-.6665
	E7-1 x Sr43	1.00	62.55500*	26.99112	.023	8.8511	116.2589
		3.00	8.18464	26.99112	.762	-45.5192	61.8885
	WT x Chinese Spring	1.00	54.37036*	26.99112	.047	.6665	108.0742
		2.00	-8.18464	26.99112	.762	-61.8885	45.5192

*. The mean difference is significant at the 0.05 level.

7.17 Annexe 17. Publication 1 from this thesis:

Arora, S., Steuernagel, B., Gaurav, K., Chandramohan, S., Long, Y., Matny, O., Johnson, R., Enk, J., Periyannan, S., Singh, N., Hatta, A., Athiyannan, N., Cheema, J., Yu, G., **Kangara, N**., Ghosh, S., Szabo, L. J., Poland, J., Bariana, H., Jones, J. D. J., Bentley, A. R., Ayliffe, M., Olson, E., Xu, S. S., Steffenson, B. J., Lagudah E., & Wulff. B. B. H. 2019. "Resistance gene cloning from a wild crop relative by sequence capture and association genetics". Nature Biotechnology 37: 139–143. https://doi.org/10.1038/s41587-018-0007-9

7.18 Annexe 18. Publication 2 from this thesis

Kangara, N., Kurowski, T. J., Radhakrishnan, G. V., Ghosh, S., Cook, N. M., Yu, G., Arora, S., Steffenson, B. J., Figueroa, M., Mohareb, F., Saunders, D. G. O. and Wulff, B. B. H. 2020. "Mutagenesis of *Puccinia graminis* f. sp. *tritici* and selection of gain-of-virulence mutants." Frontiers in Plant Science 11:1-14 https://doi.org/10.3389/fpls.2020.570180