Characterisation of major gene (*Stb*)-mediated resistance to Septoria tritici blotch disease in wheat

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Common abbreviations

Abbreviation	Full word or phrase
STB	Septoria tritici blotch
Z. tritici	*Zymoseptoria tritici*
Stb resistance gene	Septoria tritici blotch resistance gene
dpi	Days post inoculation/Days post infection
RResHT	Prefix for *Z. tritici* isolates using the naming conventions of this project
CS	Chinese spring
KASP marker	Kompetitive Allele Specific PCR marker
SSR	Single sequence repeat
RFLP	Restriction fragment length polymorphism
SNPs	Single nucleotide polymorphism
QTL	Quantitative trait loci
WAK gene	Wall associated kinase gene
Avr gene	Avirulence genes
F_3	Segregating plant population produced from hybridising two wheat lines and selfing the resulting population for three generations
NILs	Near isogenic lines
BC_3	Quasi-NIL lines produced by backcrossing a population back to one parental line for three generations
PCR	Polymerase chain reaction
cPCR	Colony PCR
OD_{600}	Optical density when measured at a 600 nm wavelength
VIGS	Virus Induced Gene Silencing
ANOVA	Analysis of Variance
dsRNA	Double Stranded Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid

Abstract

It is vital that *Stb* resistance genes are optimally used to protect elite wheat lines from *Z. tritici,* a highly adaptive fungal pathogen of wheat which has demonstrated rapid gain of virulence against many previously used *Stb* resistance genes.

In a screen testing 19 known *Stb* resistance genes against arrays of up to 90 recent UK *Z. tritici* field isolates, the resistance genes *Stb5, Stb10, Stb11* and *Stb19* were identified as providing exceptionally broad resistance. Together these genes would offer protection from all tested *Z. tritici* isolates. The origins of these genes suggest that synthetic wheats may be rich sources of novel broadly effective *Stb* resistance genes in the future.

We have also developed KASP markers capable of tracking the resistance genes *Stb5* and *Stb18,* as well as confirming such markers for *Stb10*. Smaller numbers of potential markers of varying quality have also been tested for other *Stb* genes (such as *Stb13* and *Stb14*), with varying levels of success. It is hoped that these data and tools will contribute to the breeding of elite wheat varieties containing multiple broad spectrum pathogen resistance genes for improved durability.

Finally, fluorescent *Z. tritici* strains were produced using agrobacterium-mediated transformation of isolates which possess virulence profiles of interest for future testing, using GFP and mCherry markers. Preliminary testing of these fluorescent *Z. tritici* lines against the *Stb5* resistance gene were unfortunately inconclusive, but adjustments to the procedure used have been suggested. It is hoped that using these suggestions, it will be possible to investigate whether stomatal closure is a relevant resistance mechanism in this pathosystem in the future.

It is envisaged that these outcomes from existing and future work in this project will enable the development of wheat lines protected by pyramids of *Stb* resistance genes, robustly protecting future wheat from *Z. tritici* infection.

Chapter 1- Literature review: Current and future methods to protect wheat yields from *Zymoseptoria tritici*

Abstract

Zymoseptoria tritici causes Septoria tritici blotch (STB), a severely damaging disease of wheat in European climates. Although STB has historically been controlled through the use of fungicides and numerous major host resistance genes, the pathogen is continuously adapting to these controls, resulting in the constant need for the development of new forms of protection. Currently, research necessary for the utilisation of such new protection methods is delayed by a lack of genetic and mechanistic information relating to existing *Stb* resistance genes, and due to the use of suboptimal model isolates that no longer reflect the diversity of wild strains. Recent genetic and biological tools are now enabling researchers to uncover the sequences and mechanisms of existing *Stb* resistance genes, enabling scientists and breeders to more efficiently develop increased resistance against this disease in elite wheat lines. This will be an important step towards ensuring our future food security.

1. Fungal Threats and Plant Disease Resistance

Wheat and other grain crops are vital parts of the human food chain, especially in temperate regions such as Northern Europe, accounting for up to 20% of human calorie consumption (Shiferaw *et al.*, 2013). One of the primary threats to wheat yield over much of the world is fungal disease. Biotrophic fungal pathogens can reduce crop growth rates by syphoning resources away from the plant, while necrotrophic pathogens directly kill plant cells. Many wheat breeding programs have the aim of reducing the impact of fungal diseases. However, full stable resistance has not yet been accomplished for most such pathogens.

Generally, fungal pathogen resistances are either highly specific gene-for-gene resistance that rely on the identification of specific fungal effectors, or more general resistances to a disease or set of diseases more broadly. Of these types, it is generally the broader resistances that provide more stable and complete long-term protection from diseases (and can also increase the durability of qualitative gene-for-gene resistances present in the same line, as was the case with *Rlm6* (Brun *et al.*, 2010)) as these resistances are more likely to be triggered by features or processes that are more difficult for pathogens to adapt under selection pressure. However, broader resistances often require more breeding effort to produce lines with the same level of resistance as such resistances are generally partial. The less intense infection responses of broad, quantitative resistances are favoured due to the greater possibility of the broadly conserved Pathogen-Associated Molecular Markers (PAMPs) that trigger such broad resistances being the result of the presence of benign or symbiotic microorganisms,

whereas the effectors that trigger gene-for-gene resistances are clear signs of the presence of a potentially damaging pathogen (Jones and Dangl, 2006).

It is unlikely that any host resistances will prove to be fully durable in the long term, as given appropriate time and selection pressure most fungal diseases will evolve to overcome almost any defence mechanism. Only resistances sufficiently effective to wipe out a pathogen population before such adaptations can develop will allow the permanent removal of a major crop disease as a consideration for future growers.

Additionally, as the consequences of climate change progress over the coming decades, new diseases are likely to become an issue in current high wheat-growth agro-systems such as Europe as fungal ranges change. This growing number of resistance targets will require breeders to prioritise the most significant fungal diseases, and the most effective resistances to them.

2. Symptoms and significance of STB

The necrotroph *Zymoseptoria tritici* is a highly economically and socially significant cereal pathogen worldwide, causing Septoria tritici blotch (Septoria) disease on wheat (*Triticum aestivum*). This fungal disease presents as chlorotic and necrotic tissue on the leaf surfaces of wheat plants that reduces their photosynthetic capacity, causing crop yield loss, followed by the development of small black pycnidia from which the fungus spreads once an infection reaches the reproductive stage.

Although Septoria is widespread, the most damaging epidemics occur under the mild and damp conditions common in temperate regions. Optimal conditions for maximum infection risk include temperatures in the region of 22°C or cooler, with 100% humidity in the field canopy and with infected leaves remaining damp during the 48-hour period after their initial contact with inoculant material (Ghaffary *et al.*, 2018), when the infection is most sensitive to environmental factors (likely as the fungus has not yet entered the leaf and is still exposed to the external conditions). Suboptimal conditions will usually delay the symptoms development but may not reduce the total damage done over the infection cycle or the average yield losses of the crop, particularly if conditions remain close to optimal over the initial 72-hour period after infection (Chungu *et al.*, 2001).

Due to these requirements for maximal infection and its use of wheat crops as a preferred host, the *Z. tritici* pathogen is most problematic in the heavily wheat based agricultural system of Europe (although it can also be a threat in North and South America and North Africa when

climatic conditions are suitable). Approximately €1 billion are spent on fungicides for wheat protection against Septoria in Europe each year, making up approximately 70% of all fungicides applied in this region (Duveiller *et al.*, 2007; Torriani *et al.*, 2015). This represents a considerable expenditure of resources and illustrates the scale of the issues that Septoria epidemics can cause in modern intensive farming systems.

This level of investment in Septoria protection can be justified due to the damage that a major epidemic can cause. Under disease-favourable conditions it is possible for *Z. tritici* to reduce crop yields by up to 50%. Even with the deployment of currently available field resistances in elite wheat lines and the use of fungicide spray protections traditionally used under intensive European farming practices, losses of five to ten percent due to this disease are still common, placing a considerable drain on production (Fones and Gurr, 2015). The average losses from susceptible lines (including many of the highest yielding lines) in the UK grown without fungicide protection has been estimated at around 20% (Fones and Gurr, 2015).

3. Control of STB

Most of the current protection methods used to mitigate crop losses due to STB are based on fungicide application, as only limited levels of resistance are available in current wheat elite lines and established biological control agents for this disease are currently lacking, although new biocontrol options for this disease are frequently tested and a small number do show some promise in tests (Arraiano and Brown, 2006; Eisner *et al.*, 2023). However, the variety of the chemical control options available to growers is becoming more limited worldwide (especially in the high risk wheat growing regions of Europe) due to the passing of environmental protection laws that limit or ban the use of chemical protectants, particularly those that offer protection from the broadest selections of pests and pathogens (Hillocks, 2012; Shattuck, 2021).

This loss in fungicide diversity is likely to be particularly problematic when considering *Z. tritici* in comparison to many fungal diseases due to the capacity of this pathogen to carry out sexual reproduction, together with the high flexibility of the pathogens genome structure (including high numbers of transposable elements composing over 18% of the genome, and up to eight accessory chromosomes some of which can be lost altogether in some lines (Habig, Lorrain, *et al.*, 2021)). This process maintains high genetic diversity in *Z. tritici* populations, even over a small geographic area, and allows the reshuffling of genetic combinations between generations. Such reshuffling aids the adaptation of the species to new conditions,

including the presence of fungicides, allowing for the rapid emergence and spread of fungicide resistances and the collection of these resistances in multi-fungicide resistant strains (Cools and Fraaije, 2008; van den Berg *et al.*, 2013). Recent studies suggest that between 28 and 130 million pycnidiospores per hectare may be released carrying adaptive mutations for fungicide resistance or virulence on resistant wheat varieties each year during epidemics (McDonald *et al.*, 2022). Such high levels of resistant mutants being produced each generation suggests that most wheat fields may contain sufficient standing genetic variation that some isolates will already be equipped to overcome each new STB control method even before the initial use of that method. Resistant isolates have been identified for each fungicide class currently in use against this disease (Bartlett *et al.*, 2002; Estep *et al.*, 2015; Vestergård *et al.*, 2023).

Such resistance can become widespread for widely utilised fungicides, drastically reducing their effectiveness in crop protection. Key examples of this include quinone outside inhibitors (QoIs) and demethylase inhibitors (DMIs, also known as azoles), both of which have traditionally been effective against *Z. tritici* (Stammler and Semar, 2011).

QOIs target the mitochondrial respiratory pathways of fungi by blocking electron flow through the cytochrome bc1 complex. This requires them to bind to the Qo site of the complex (Drabešová *et al.*, 2013). Mutations to the cytochrome b protein that render these inhibitors ineffective emerged quickly after the fungicide first entered use in 1996 (Bartlett *et al.*, 2002). The most common mutation of this kind is G143A, in which an alanine amino acid residue is replaced with glycine in this protein, preventing the fungicide's intended interaction (Fraaije *et al.*, 2005; Lavrukaitė *et al.*, 2023). This mutation was first found in Europe (likely due to the intensive farming methods and agricultural focus on wheat in the region) in 2001, and has been estimated to have emerged independently on at least four occasions and spread rapidly (Estep *et al.*, 2015). Resistant fungal strains of European origin have since been reported in North America where they spread rapidly through the native population (Estep *et al.*, 2013), aided by the active sexual reproduction of *Z. tritici*.

DMIs function by inhibiting the fungal 14α-demethylase enzyme (coded for by the gene *CYP51*), which targets lanosterol and 24-methylene-24,25-dihydrolanosterol during fungal sterol production (Gisi *et al.*, 2000). Several mutations have been identified that can reduce the effectiveness of these fungicide treatments via at least 22 distinct amino acid alterations in CYP51 (Estep *et al.*, 2015). A general increase of the EC_{50} (the dose required to reduce the rate of pathogen growth by 50% in vitro) of European *Z. tritici* isolates to DMIs has been identified (due primarily to mutations in the CYP51 enzyme, but also

linked to the upregulation of efflux transporters such as the ABC and MFS transporters that can reduce fungicide build-up (Estep *et al.*, 2015)). Levels of these mutations are rising around the world, with resistances becoming almost fixed in some populations (e.g. 97% of one Oregon *Z. tritici* population were found to have resistance-related CYP51 mutations in 2015 (Estep *et al.*, 2015)).

Although the field is relatively new and not yet widely used, RNAi treatments have also been tested for their ability to suppress *Z. tritici* development. This system involves using small double stranded RNAs to silence specific fungal genes (from these Dicer RNase III generates 20-30 nucleotide lengths of dsRNA, which is then bound by Argonaute proteins in the RNA induced silencing complex (RISC), which uses a single strand of the dsRNA as a guide to identify matching RNA sequences for degradation). Although previous studies have suggested that RNAi pathways are not directly involved in virulence in *Z. tritici* (Kettles *et al.*, 2019; Ma *et al.*, 2020), more recent data suggests that conserved RNAi machinery in this fungal species may be involved in asexual reproduction and therefore relevant to the progression and spread of disease (Habig, Schotanus, *et al.*, 2021). Direct RNAi treatments and Host-Induced Gene Silencing (HIGS) have proven ineffective for controlling *Z. tritici,* likely because the pathogen is not capable of the efficient dsRNA uptake required for gene silencing to be initiated (Kettles *et al.*, 2019).

4. Infection and life cycle of *Z. tritici*

When control methods for *Z. tritici* fail or are not deployed, the disease can spread rapidly through wheat fields using splashes from rainfall to spread spores to new leaves in the canopy. Near the beginning of the wheat growing season, ascospores will be released from pseudothecia that have overwintered on stubble and other wheat remains from the previous harvest – these will reproduce sexually on wheat leaves to produce new infections. As infections spread throughout the growing season, pycnidiospores released from pycnidia growing on plants in advanced stages of the infection will become the primary inoculum generating new infections (Eriksen and Munk, 2003; Steinberg, 2015).

These ascospores and pycnidiospores will quickly germinate to produce hyphae that enter the leaves through stomata, but will not cause immediate symptoms, making it difficult to identify and treat infected areas quickly in the field. This symptomless or latent period lasts for at least eight days (generally longer on more resistant wheat genotypes, up to approximately 14 days), during which fungal growth is confined to slow hyphal extension. During this phase the fungus grows in the apoplastic space, but does not cause necrosis or trigger

hypersensitive disease responses in the host (Sánchez-Vallet *et al.*, 2015).

This early asymptomatic phase was traditionally considered a biotrophic stage in the fungal life cycle (leading to *Z. tritici* being commonly classified as a hemibiotroph). However, the lack of fungal feeding structures combined with the presence of fungal lipid metabolism related metabolites in metabolomics studies at this infection stage (including 10 of the 38 metabolites that are present in increased concentrations during this period) suggests that most of the fungal nutrition used for this growth is probably drawn from stores in the original spore and not from the plant host (Rudd *et al.*, 2015). This conclusion is supported by the fact that at one day post infection the fungus had an increased prevalence of protein annotations involved in the ß-oxidation of fatty acids, and that genes involved in the transport and ß-oxidation of such acids were upregulated between one and four days post infection, as were malate synthase and isocitrate lyase (two important glyoxylate cycle enzymes involved in the use of fatty acid derived acetate) (Rudd *et al.*, 2015). The apparent lack of fungal biomass increase during this phase also supports the theory that the fungus survives on its own reserves, with disease resistance levels of the host plant not significantly affecting the biomass accumulation (as measured through quantity of DNA present) of *Z. tritici* isolates until after the end of the shortest latent periods at 11 days post infection (Rahman *et al.*, 2020).

Although the purpose of this latent phase is not well understood, it appears to be related to later reproductive stages. The order of *Z. tritici* life cycle stages and their importance to the fungal reproductive cycle is described in Figure 1. Several sexual reproduction-related genes are upregulated only in the latent phase and the silencing of wheat genes such as *TaR1* led to both a reduced latent phase and a reduction in the later asexual fecundity of silenced isolates (J., Lee *et al.*, 2015).

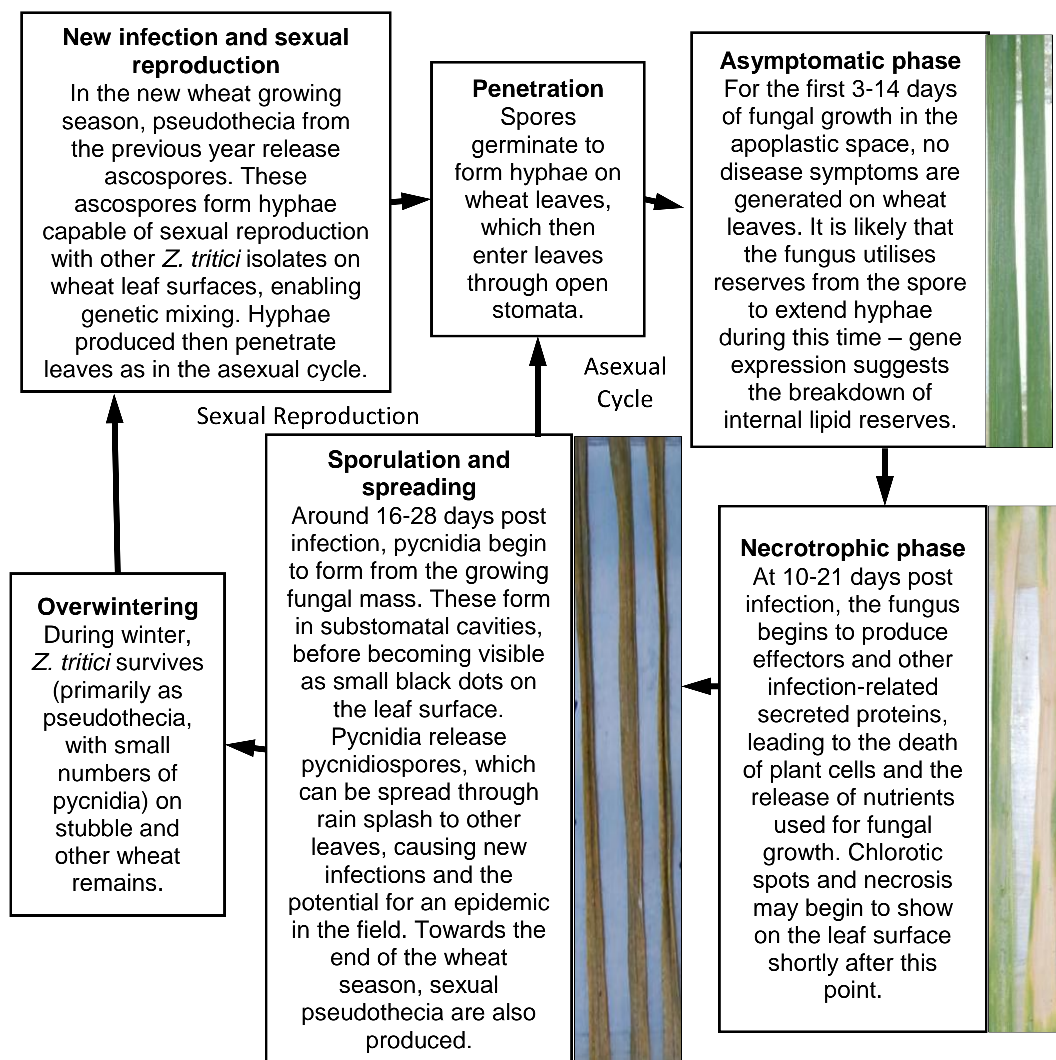

Figure 1: Flowchart showing the lifecycle of *Z. tritici* epidemics in the field. Images from research described in Tidd *et al.*, 2023 are used where appropriate to illustrate the infection symptoms visible on wheat leaves.

To remain undetected through the asymptomatic stage, the fungus uses effectors to supress plant immunity. For example, the effector Mg3LysM is broadly present in the *Z. tritici* population, and appears to supress plant immune responses by binding chitin released from the fungal cell wall (which the plant would otherwise detect as a pathogen-associated molecular pattern (PAMP)) and inhibiting the breakdown of cell wall chitin by plant chitinases (Marshall *et al.*, 2011; Sánchez-Vallet *et al.*, 2015). Mutant fungal strains in which this gene has been rendered non-functional are severely affected in their ability to infect wheat, but can be rescued in wheat leaves in which the chitin receptor genes *CERK1* or *CEBiP* are silenced (Lee *et al.*, 2013).

These initial immunity suppression effectors are accompanied by overlapping series of phase specific effectors (Rudd *et al.*, 2015). Many fungal secreted proteins (likely including many effectors) start to

be upregulated between four and nine days post inoculation (Rudd *et al.*, 2015), and the manipulation of H_2O_2 production may also play a role in protecting *Z. tritici* from detection by the host immune system (Shetty *et al.*, 2007; Rudd *et al.*, 2015; Orton *et al.*, 2017; Brennan *et al.*, 2019). Light-dependent production of superoxides by *Z. tritici* and changes in the host regulation of photochemical quenching may also be relevant to the regulation of non-specific host defences, and that of the specific chlorotic and necrotic effects induced on wheat leaves. However, wider testing is required to confirm whether such responses to infection are consistent among susceptible and resistant wheat lines (Ajigboye *et al.*, 2021).

Although the primary purpose of immune suppression is presumed to be enabling the growth of the original *Z. tritici* colony, recent research suggests that this may cause systemic induced susceptibility during the latent phase which could enable secondary infections to colonise the plant more easily (Seybold *et al.*, 2020). This could be evolutionarily advantageous to *Z. tritici* as its spores are released over an extended period (allowing early virulent infections to leave wheat plants more vulnerable to infection by later spores through the suppression of host defences, potentially acting as a kind of kin selection). However, the high diversity of *Z. tritici* populations combined with the possibility of other pathogens also benefiting from this induced susceptibility make it difficult to predict whether the benefits of this system actually outweigh the costs of increased competition from more distantly related *Z. tritici* isolates or other, unrelated pathogens.

As the fungus continues to grow in the apoplast of infected wheat leaves, it transitions to a necrotrophic feeding habit to meet the growing energy and biomass needs of the fungal colony. During this stage, plant immunity is suppressed and effector-driven cell necrosis begins (making nutrients available to *Zymoseptoria*). This generally occurs within 10-21 days of initial infection, with longer latent periods generally associated with more resistant host plants.

As the symptomatic phase begins, chlorotic spots will appear on infected leaf surfaces as host defence mechanisms are activated. As the infection progresses, necrosis symptoms will begin to develop on the leaves of susceptible cultivars. This is accompanied by an increase in fructan metabolic flows in the plant, possibly stimulated by the fungal effectors to provide nutrients to the fungus or as part of the plant's stress response to fungal growth and damage caused by other effectors (Rudd *et al.*, 2015). There is also a release of plant cell wall attacking enzymes as the infection progresses to full necrosis between 14 to 21 days after infection, along with increases in fungal transporters and digestive enzymes, allowing the *Z. tritici* colony to feed from the dead plant tissue (Rudd *et al.*, 2015). In a compatible interaction, this causes widespread necrosis on the leaf surface.

This increase in the availability of fungal nutrition with the onset of widespread necrosis in host tissue leads to a spike in fungal growth rates. This enables the development of fungal asexual reproductive structures or pycnidia, initiating from approximately 16-28 days after inoculum exposure. Asexual pycnidia are responsible for most of the growth and spread of Septoria epidemics within a wheat growth cycle (Ghaffary *et al.*, 2018) as the absence of a need for fertilisation allows the pycnidiospores within to be developed and released quickly, and to begin the process of germination and penetration through stomata immediately after infection. Most of the spread of infection through this method occurs during the highest precipitation periods of the wheat growth cycle, because rain splash is required to carry pycnidiospores between wheat leaves. This requirement is also believed to be the primary reason why distance between leaves in wheat plants correlates positively with wheat STB resistance, as there is less chance of inoculum being transferred over larger distance between more distant leaves.

At 25 to 30 days post infection, near the end of the wheat growth cycle, the fungus begins to develop sexual pseudothecia on the dead host tissue (Sánchez-Vallet *et al.*, 2015). These pseudothecia will overwinter on wheat stubble or other host detritus between wheat growth seasons, with the ascospores released from them being spread by rain splash to new wheat plants near the beginning of the next wheat growth cycle.

The hyphae produced from pseudothecia carry out the act of reproduction between fungal isolates where such hyphae meet on wheat leaves, helping to maintain genetic diversity within populations and to shuffle alleles and mutations between isolates. This enables *Z. tritici* populations to adapt rapidly to changing conditions, such as gaining resistance to a widely used fungicide or virulence against a single major host resistance gene, which a single fungal mutation could provide resistance to. This sexual reproduction contributes heavily to *Z. tritici*'s ability to break many resistance genes in wheat quickly and is a major concern given that a small number of fungicides are currently used to protect most European wheat from the heavy losses that Septoria can inflict. For most of these fungicides, resistance already exists in the *Z. tritici* population (Torriani *et al.*, 2015) and sexual reproduction could allow these resistances to spread, potentially forming isolates with multiple fungicide resistances given sufficient selection pressure.

The lifecycle of *Z. tritici* presents multiple difficulties to growers. Infection takes place through different routes from both infected wheat (through pycnidiospores) and old wheat material often left in fields to maintain soil quality (through ascospores). Pseudothecia enable the disease to linger in legacy material throughout what would otherwise

be unsurvivable environmental conditions, making the fungus hard to remove from populations, while pycnidia enable the fungus to reproduce quickly and thus spread rapidly during growing seasons. The latent phase of infection cycles makes the disease difficult to treat efficiently with targeted fungicide applications, while the sexual reproductive cycle of the pathogen enables it to adapt rapidly to new protections. These issues make STB difficult to deal with through alterations to farming methodologies, while bio-protections from this disease are not widely available and the effectiveness of fungicide protections cannot be considered fully reliable. It is therefore important that host resistances to this disease are well understood and utilised in the field as our most efficient and sustainable mechanism of control.

5. Current Limitations to the research and utilisation of *Stb* Genes

Resistance to *Z. tritici* is a relatively new target in wheat breeding, meaning that much of the research relating to this pathogen and its interactions with crop plants is still in the early stages. However, in addition the identification of several host resistances, recent evidence suggests that small secreted proteins causing transcriptional changes are likely to be relevant in causing leaf necrosis (Welch *et al.*, 2022), and that effectors such as Mg3LysM, Mg1LysM and Mgx1LysM are important for the fungus to avoid PAMP-triggered plant immune responses (Tian *et al.*, 2021).

Much of the research conducted thus far into this pathosystem has utilised the model strain isolate held by most laboratories, IPO 323. However, this isolate is now outdated compared to modern field populations, as it is naïve to all modern fungicides and avirulent on cultivars with disease resistance genes such as *Stb6* that have now been broken down by a large majority of isolates found in the field. There are also concerns that this isolate may have diverged genetically in different labs due to many cycles of replication on agar plates, producing new selection pressures and providing time for genetic drift to occur (the potential for which is demonstrated in directed evolution experiments, such as (Fouché *et al.*, 2021)). The use of this isolate as a model for *Z. tritici* can make it difficult to standardise and compare work done using more recent isolates that may differ substantially from IPO 323 genetically but which provide a better representation of fungal genotypes present in a modern wheat field.

Field trials can also be difficult to standardise due to genetic differences in *Z. tritici* populations across the world or even regionally in the same country (e.g. different levels of fungicide resistance) and the dramatic effect of weather (particularly rainfall) on Septoria levels

causing large fluctuations in readings between years (Ouaja *et al.*, 2020). Additional complexities are added to data analysis by wheat lines with resistance levels that change over the wheat life cycle (e.g. high seedling and low adult resistance (Tabib Ghaffary *et al.*, 2012)) and by imperfect correlations between the levels of different symptoms (e.g. necrosis levels and pycnidia counts (Ouaja *et al.*, 2020)).

The currently limited availability of data on the interaction between *Z. tritici* and wheat, along with the difficulty in comparing data from different sources, is problematic as it has limited our ability to identify sources of quantitative resistances to this disease and our understanding of the full range of variation in *Z. tritici* and the conditions to which isolates of this pathogen can be adapted (Chartrain, Brading, Widdowson, *et al.*, 2004; Boixel *et al.*, 2022). This, combined with the fact that few wheat lines have a history of being bred for Septoria resistance, has led to a dearth of lines with significant quantitative resistance to the disease, leaving most growers reliant on fungicides and major resistance genes to protect their yields (Torriani *et al.*, 2015). Unlike those for traditional breeding targets such as rust diseases, most of the few broad resistances that are available for *Z. tritici* have yet to be bred into the large majority of elite lines. Newer resistances such as *Stb16q* can have broader effects than older resistances (Brown *et al.*, 2015; Kettles and Kanyuka, 2016), but virulent *Z. tritici* isolates develop within a few years and propagate through the population, reducing the effectiveness of these resistance genes against field populations of the pathogen (e.g. the breaking of the *Stb16q* resistance in Ireland and Iran (Dalvand *et al.*, 2018; Kildea *et al.*, 2020)). Only lines containing multiple resistance genes (e.g. Kavkaz-K4500, which contains four known major resistance genes) seem to maintain broad spectrum resistance in the medium to long term (Chartrain, Berry, *et al.*, 2005; Mundt, 2014).

The reliance upon major host resistance genes for protection against STB can leave agricultural systems vulnerable to emerging virulent pathogen strains – for example, the breaking of the resistance found in the cultivar Gene. Gene was grown primarily in the USA, and was fully resistant at its release in 1992, but this resistance was widely lost by 1995, causing substantial crop losses as few other resistances were present to mitigate the damage (Cowger *et al.*, 2000). A similar issue occurred with the breakdown of the resistance associated with the cultivar Cougar in the UK in 2015 (only two years after being originally placed on the UK recommended list), a breakdown that also affected the field resistance of multiple varieties descended from Cougar, which was used as a resistance source in multiple breeding programs (Kildea *et al.*, 2021). This resistance breakdown problem will only increase as effective fungicide protection options become more limited (Birr *et al.*, 2021).

It is therefore clear that a future priority in wheat breeding is likely to be the development of elite lines containing a greater variety of disease resistance genes. Major resistance genes are likely to be a large part of this as they can be identified easily and applied quickly in breeding programs, and those genes not yet broken by a *Z. tritici* population will provide excellent field resistance in that region. Quantitative resistance genes may provide broader and more durable resistance, but the partial resistance each such gene provides is of less immediate interest to most breeders and growers, and breeding for such resistances without excellent markers is far more difficult due to the need to accurately phenotype plants that may carry multiple partial resistances. Suitable markers for such breeding are also more difficult to develop for such partial resistances due to similar issues with phenotyping large datasets (particularly with a pathogen that shows as much environmental variation in its symptoms as *Z. tritici*). For these reasons, most of the widely used resistance genes against Septoria tritici blotch are major gene-for-gene resistances.

More than 20 such major *Stb* (*Septoria tritici blotch*) resistance genes that could be used in wheat breeding programs have thus far been identified, providing natural protection against a variety of *Z. tritici* isolates at the different stages of the wheat life cycle (referred to as seedling, adult or seedling and adult resistance genes) (Dreisigacker *et al.*, 2015). For many of these *Stb* genes we have some information relating to their chromosomal locations, but in the majority of cases this data is imprecise.

6. Known *Stb* (*Septoria tritici blotch*) resistance genes

The currently known disease resistance genes available from wheat stocks include *Stb1, Stb2, Stb3, Stb4, Stb5, Stb6, Stb7, Stb8, Stb9, Stb10, Stb11, Stb12, Stb13, Stb14, Stb15, Stb16q, Stb17, Stb18, Stb19, StbWW, TmStb1* and *StbSm3*. These resistance genes have been identified from a variety of sources, with some being easier to transfer into modern breeding programs than others. This data is summarised in Table 1.

Although many *Stb* genes are present to some extent in elite lines, it is likely to be novel resistances from landraces and the wheat secondary gene pool (including ancestor species and synthetic wheats) that are the most durable and broadly effective when widely applied, as their previous absence from agriculture has left little selection pressure, driving current *Z. tritici* populations to adapt to them and gain virulence. Unfortunately, these resistance genes will also be the most difficult to move to elite lines due to potential yield drag and reproductive barriers (e.g. ploidy numbers, particularly regarding the ancestor species).

Table 1: Summary of the currently available *Stb* resistance genes available in wheat, and their likely values and limitations in a breeding program.

Name of *Stb* gene	Approximate chromosomal location	Wheat line in which *Stb* gene was identified	Paper reporting discovery	Use, breeding and other notes
Stb1	5B (Long arm)	Bulgaria 88	(T B Adhikari *et al.*, 2004)	Widely bred into soft red winter wheats including Oasis and Sullivan as early as 1975 (Goodwin, 2007), widely used in North America until 1990's. Now widely broken.
Stb2	1B (Short arm)	Veranopolis	(Liu *et al.*, 2013)	Found in elite wheat cultivars.
Stb3	7A (Short arm)	Israel 493	(Goodwin *et al.*, 2015)	Available in breeding programs.
Stb4	7D (Short arm)	Tadinia	(Tika B Adhikari *et al.*, 2004)	Could be easily moved into modern breeding programs.
Stb5	7D (Short arm)	Synthetic 6X	(Arraiano *et al.*, 2001)	Located near centromere in synthetic wheat lines – likely to be linked to undesirable traits with low recombination rate (Akhunov *et al.*, 2003). Has been tested in Chinese Spring background, but not widely used in breeding programs (Arraiano et al., 2001; Ruth Bryant, personal communication). Likely to be difficult to access in breeding programs.
Stb6	3A (Short arm)	Flame	(Brading *et al.*, 2002)	Widely used in Europe in varieties including Hereward, Shafir, Bezostaya 1 and Vivant. Has been cloned and sequenced (Saintenac *et al.*, 2018). However, now widely broken in Europe (Stephens *et al.*, 2021).

Stb7	4A (Long arm)	ST6 population (selection drawn from Estanzuela Federal)	(McCartney *et al.*, 2003)	Relatively easy to move into modern breeding programs.
Stb8	7B (Long arm)	Synthetic hexaploid population W7984	(Adhikari *et al.*, 2003)	Similar circumstances and issues to *Stb5*
Stb9	2B (Long arm)	Courtot and Tonic	(Chartrain *et al.*, 2009)	Relatively easy to move into modern breeding programs. The relevant avirulence gene has also been reported (Amezrou *et al.*, 2022).
Stb10	1D (Near centromere)	Kavkaz-K4500	(Chartrain, Berry, *et al.*, 2005)	Near centromere, low recombination rates may be issue.
Stb11	1B (Short arm)	TE9111	(Chartrain, Joaquim, *et al.*, 2005)	Already present in breeding lines.
Stb12	4A (Long arm)	Kavkaz-K4500	(Chartrain, Berry, *et al.*, 2005)	Close to *Stb7*. Shown to be distinct in inheritance studies, but closely linked.
Stb13	7B (Near centromere)	Salamouni	(Cowling, 2006)	Possibly sharing a common ancestor with *Stb4* and/or *Stb5*. Present in landraces, may require some pre-breeding.
Stb14	3B (Short arm)	Salamouni	(Cowling, 2006)	Close to resistance gene cluster covering many fungal pathogens – any adaptive benefit from this is currently unclear. Present in landraces, may require some pre-breeding.
Stb15	6A (Short arm)	Arina	(Arraiano *et al.*, 2007)	Could be directly used in breeding programs, but seems to provide limited field resistance (Arraiano *et al.*, 2009; Brown *et al.*, 2015).

Stb16q	3D (Long arm)	Synthetic M3	(Tabib Ghaffary *et al.*, 2012)	Derived from synthetic wheat, present in chromosome region with repressed recombination. Broadly effective in seedling and adult plants, has been cloned as a cysteine-rich receptor-like kinase. (Saintenac *et al.*, 2021). However, this resistance has already been broken by *Z. tritici* in Ireland (Kildea *et al.*, 2020), Iran (Dalvand *et al.*, 2018) and France (Ruth Bryant, personal communication)
Stb17	5A (Long arm)	Synthetic M4	(Tabib Ghaffary *et al.*, 2012)	Identified in synthetic wheat, but with higher recombination rates then *Stb16q*. Pre-breeding still required, but less extensively.
Stb18	6D (Short arm)	Balance	(Tabib Ghaffary *et al.*, 2011)	Relatively easy to move into modern breeding programs.
Stb19	1D (Short arm)	Lorikeet	(Yang *et al.*, 2018)	Identified in wheat of synthetic descent. Novel resistance, not yet widely used, should be deployed with protection.
StbWW	1B (Short arm)	Advanced breeding lines WW1842, WW2449 and WW2451	(Raman *et al.*, 2009)	May be allelic to *Stb11* – not yet confirmed experimentally (Raman *et al.*, 2009; Dreisigacker *et al.*, 2015). Also located near Stb2 and several QTLs (Piaskowska *et al.*, 2021), may form a linkage group. Effective in seedling and adult plants, has been identified in several breeding lines, so likely already present in breeding programs.

StbSm3	3A (Short arm)	Salamouni	(Cuthbert, 2011)	Identified in landraces – likely to require some pre-breeding.
TmStb1	*Triticum monococcum* 7A (Short arm)	*Triticum monococcum*	(Jing *et al.*, 2008)	Identified in *Triticum monococcum*, (a diploid wheat species, with an A^m genome related to the wheat A genome). May provide high *Z. tritici* resistance but would take significant effort to introgress before true breeding efforts could begin.

A possible solution to the need for pre-breeding is the use of genetic engineering and gene editing technologies to move desired alleles quickly and precisely between different lines, without transferring unwanted genes and regardless of any reproductive barriers between the lines concerned. This would allow cloned resistance genes to be easily pyramided in new cultivars with tight linkages to one another by inserting multiple resistance genes into a small area of the wheat genome, making it easy to monitor and maintain these genes together in a breeding program. However, public acceptance of genetic manipulation technologies is an ongoing issue that prevents their adoption across much of the world.

Although the long term economic and humanitarian benefits of being able to utilise modern techniques to create superior plant products are huge (due to the health benefits and increased production possible with lower inputs and less economic risk to the grower), the short term gains are generally viewed as insufficient to justify expending political capital on by most political groups, and significant opposition to the use of this technology exists among less scientifically literate groups in the environmental community (Dancer and Shiel, 2019). It is therefore unlikely that these techniques will be available for use in the production of Septoria resistant wheat varieties for the foreseeable future, especially in the highly wheat-focused agricultural system of Europe (even in nations such as the UK, which is likely to reassess such regulation in the near future due to the political disruption of Brexit).

Overcoming this public distrust of genetically manipulated food would require support from political and regulatory bodies (as in many areas legislative changes would be required to make products produced in this way commercially viable). Generating the political will to implement these changes is likely to require large scale campaigns to raise epistemic trust (trust in the underlying science behind this technology) by emphasising the evidence that products produced using modern

genetic manipulation techniques are not inherently dangerous to human health or the environment, along with clearly communicating both the individual and social benefits that can be achieved using this technology over conventional breeding. Research suggests that independently of trust in the underlying technology, trust in public institutions may be more closely linked to perceptions of the benefits of these technologies, while trust in private/industrial organisations may be more closely linked to perceptions of the associated risks (Hu *et al.*, 2020). This may suggest that the optimal ways to encourage public uptake of these foods may differ for different organisations, although it also seems likely that high trust in the integrity and competence of public regulatory bodies will increase public trust in the private corporations against which this regulation is enforced.

An additional challenge to most modern breeding methods for Septoria resistance, especially high precision methods such as genetic modification, is the fact that only *Stb6* and *Stb16q* have been cloned (along with the cognate fungal effector AvrStb6 recognised by *Stb6*) (Zhong *et al.*, 2017; Saintenac *et al.*, 2018; Saintenac *et al.*, 2021). Of these genes, *Stb6* has been widely broken in the UK and although *Stb16q* still provides relatively broad resistance, *Z. tritici* isolates virulent against wheat containing this resistance have now been identified (Dalvand *et al.*, 2018; Kildea *et al.*, 2020). The lack of knowledge of nucleotide sequences for other *Stb* resistance genes makes it more difficult to design molecular markers to follow *Stb* resistance genes through breeding programs (as markers cannot be designed that lay inside or very close to each gene when only the general genetic location is known). This is particularly challenging when identifying multiple resistances present in the same breeding line (e.g. *Stb10* and *Stb12* were found in Kavkaz-K4500, which also contains the previously known resistance genes *Stb6* and *Stb7*). This lack of knowledge also makes it impossible to use genetic manipulation to precisely move a desired resistance allele from one wheat line to another.

An additional complication in attempts to produce elite lines with good Septoria resistance is the fact that many of the *Stb* resistances are already partially or fully broken by different *Z. tritici* isolates, and that the populations from which these isolates are drawn may be found all over the world. This means that a resistance gene effective in one area may be ineffective in another, and a resistance breaking isolate could theoretically spread rapidly with the movement of people, machinery and crop products around the world if driven by appropriate selection pressures. This could result in wheat lines containing resistance genes that seem functional in one country being introduced to Septoria strains that are virulent against them elsewhere, leading to these virulent

Septoria strains becoming dominant or genetically fixed in their local areas and spreading to other areas.

This provides an additional reason why gene pyramiding may be desirable when attempting to breed Septoria resistant wheat lines, and may partially explain why the crop varieties and breeding lines with the most durable resistances to *Z. tritici* are usually found to contain multiple resistance genes (as it is less likely that any single *Z. tritici* isolate will develop the multiple simultaneous mutations necessary to gain virulence, making it less likely that virulent isolates will develop and spread) (Chartrain *et al.*, 2004a). For example, Kavkaz-K4500 is one of the most durable sources of field resistance used for breeding elite lines in the UK and has been shown to possess at least four qualitative resistance genes, including *Stb6*, *Stb7, Stb10* and *Stb12* (Chartrain *et al.*, 2005a). Kavkaz-K4500 was bred from the Russian winter wheat Kavkaz and the Brazilian wheat Frontana, and was introduced to CIMMYTs resistance breeding programs in the 1970s (Chartrain, Berry, *et al.*, 2005). Unfortunately due to this heritage and its age, it is not efficient to grow Kavkaz-K4500 itself in the UK compared to current elite lines, but the lines field resistance is still of great interest. This resistance is sufficient to make Kavkaz-K4500 resistant to Septoria under field conditions despite the fact that many individual *Z. tritici* isolates are pathogenic on it in laboratory tests (Chartrain *et al.*, 2004a; Chartrain *et al.*, 2005a).

It is also noteworthy that some individual major resistance genes that have been widely used in breeding so far have proved more durable than others. For example, *Stb1* was introduced to the USA grower market in the cultivar Oasis in 1975 and has been used in many other cultivars (e.g. Sullivan) since 1979, and remained effective in the field up until mid-2000's (Cowger *et al.*, 2000; Adhikari *et al.*, 2004; Singh *et al.*, 2016). *Stb4* also proved to be fairly durable, lasting for ~ 15 years. After its introduction to breeding programs in the USA in 1975 (in a cross between Tadorna, Cleo and Inia 66), the *Stb4* containing variety Tadinia underwent a commercial release in 1984 (Somasco *et al.*, 1996), and this resistance gene remained effective until 2000 (Jackson *et al.*, 2000). *Stb6* has historically been the primary STB resistance used in Western Europe, and likely entered wheat breeding programs from multiple sources before modern formalised breeding programs (Chartrain, Brading, *et al.*, 2005; Dutta *et al.*, 2021). *Stb6* is known to have been present alongside *Stb4* in the cultivar Gene, released in 1993 (Krenz *et al.*, 2008). Unfortunately the resistance of this line broke down approximately two years after the release of Gene, before the spread of virulent genotypes could be easily tracked.

These higher durability cases may be due to reduced selection pressure on *Z. tritici* to break each of these resistances specifically, due to the wider range of *Stb* genes used to protect wheat grown after

their introductions, leading to a reduced rate of fungal evolution and virulence gain. It is also possible that unknown resistance genes or QTLs present in the same lines as the more durable *Stb* genes produced additional barriers for *Z. tritici* infection, resulting in a greater delay before virulent isolates developed against these *Stb* genes, as multiple simultaneous mutations may be required to gain virulence against wheat lines containing them. Another explanation may be that to adapt to these resistances, fungal isolates were forced to sacrifice important effectors, thus reducing their ability to compete on susceptible lines. If this is the case, it is possible that gene pyramiding could be an even more effective method of Septoria control than previously thought, as fungal isolates virulent against plants with multiple resistances of this nature would be significantly less aggressive. In this way, some major gene resistances may have a secondary effect functionally similar to durable quantitative resistances.

This may explain why some quantitative trait loci (QTLs) for Septoria resistance co-segregate with major (qualitative) resistance genes such as *Stb6* (Brown *et al.*, 2015). However, most do not (and presumably interact with different parts of the plant immune system, such as the PAMP triggered immunity), often making such partial resistances difficult to find. This is problematic, as the trend observed in most pathosystems is that broad spectrum partial resistances are typically far more durable then the major gene mediated resistances currently relied upon. This is largely because several such broad spectrum resistances may be necessary to achieve high levels of resistance, and both identifying such resistances genetically and using them in breeding programs is generally more challenging than using gene-for-gene resistances, thus reducing commercial utilisation of quantitative resistances.

Generally, large studies using segregating populations are needed to provide the statistical power required for the identification of Septoria resistance QTLs, and even this method is not fully effective as partial resistance is often dependent on many minor genes spread throughout the wheat genome, making the effect of each one difficult to separate out. This seems to be the case in the resistant wheat cultivar Arina (Chartrain, Brading, Widdowson, *et al.*, 2004; Samavatian *et al.*, 2023). Additionally, many QTLs associated with resistance may also be associated with other important traits (e.g. Septoria infection is often negatively correlated with heading date and internodal distances), often indicating disease escape rather than true resistance – this is less useful as generally these linked traits will not be desirable in elite cultivars (Chartrain *et al.*, 2004b).

However, studies have uncovered potentially useful QTLs that could be tracked and used in breeding programs to build up higher levels of partial resistance in elite wheat lines. Nine such QTLs were found in

the cultivar Florett and six in Tuareg by Risser *et al.* (2011), although the analysis from this study was further complicated by the fact that most of the resistance QTLs found had environmental interactions that made their effects inconsistent. One small effect and inconsistent QTL was also identified in Biscay, the susceptible parent of the segregating populations, demonstrating that resistance QTLs can be relatively minor and difficult to identify, and that even susceptible cultivars could contribute such resistance QTLs (Risser *et al.*, 2011). Four genomic positions capable of limiting the degree of necrosis induced by *Z. tritici* infection and thirteen capable of restricting fungal asexual reproduction have also been identified across the wheat genome by Yates *et al.* (2019). Ten significant markers were identified in Nordic winter wheat using genome-wide association studies (GWAS) by Odilbekov *et al.* (2019), who also produced a genome prediction model with 0.62 prediction accuracy by using these markers as fixed effects. More recently, 37 QTLs for *Z. tritici* resistance and 29 marker-trait associations corresponding to putative genes have been identified across 17 chromosomes in 185 bread wheat genotypes by Mahboubi *et al.* (2021), and five new potential resistance QTLs have been identified using GWAS along with 14 previously identified QTLs by Mahboubi *et al.* (2022). This demonstrates that models for wheat *Z. tritici* resistance can be produced from genomic data to help improve breeding efficiency.

Overall, more work is necessary to conduct large screens that will be needed to identify resistance QTLs in wheat lines despite their relatively small individual effects. Conducting these screens on more diverse germplasm (particularly non-elite landraces and ancestor species) may help to identify new QTLs with larger effects, while using more closely related lines (e.g. producing backcrossed populations with a high degree of relatedness) may allow smaller effect QTLs to be identified by removing background noise and variation. Until such work can be conducted and the lengthy process of introgressing enough QTLs to provide broad *Z. tritici* resistance to elite wheat lines can be completed, gene-for-gene *Stb* resistances will be required to control this disease and protect wheat yields.

7. Current Research and Technological Improvements for Assisting Selection and Breeding

To support further breeding efforts for *Z. tritici* resistance, improved methods for tracking disease resistance genes not currently present in elite lines through wheat breeding programs is a priority.

The genetic markers originally developed for most *Stb* genes use outdated technology (e.g. microsatellite markers or restriction fragment

length polymorphism (RFLP), which require a polyacrylamide gel to be run each time the marker is used), limiting the effectiveness of breeding programs (Zhan *et al.*, 2003). More modern systems, combined with libraries of available single nucleotide polymorphism (SNP) derived markers, are now improving the speed and efficiency of selection programs (e.g. the Axiom array dataset, used to validate 921705 putative varietal SNPs in a 475 accession screen (Winfield *et al.*, 2016), identifying 546299 useful SNP probes, which shows the potential of this system for high throughput work).

For example, Kompetitive Allele Specific PCR (KASP) markers provide a cheap, efficient screening mechanism using a mix of fluorophore bound primers that compete for DNA binding positions and common quencher-linked complimentary primers (Dreisigacker *et al.*, 2015). This allows KASP markers to be easily targeted to any SNP or other genetic difference (such as deletions and insertions) between two lines (allowing them to be very closely linked with desired genes) and enables them to differentiate between the two homozygous and the heterozygous genotypes (which is particularly helpful in small screens where a limited amount of the desired genetic material may be available, as heterozygotes can be kept for further breeding). Such markers are especially useful for breeding adult plant disease resistance genes into elite lines, as using genetic markers allows resistance genes to be tracked in a population using simple PCR reactions, rendering it unnecessary to grow plants to maturity and phenotypically test them for resistance.

Avoiding the necessity for phenotyping is a drastic improvement compared to older breeding programs, as phenotyping often limits the power of breeding programs in which such a step is required (as well as potentially introducing inaccuracy and bias) due to the time consuming and often subjective nature of by-eye symptom assessments. These difficulties are compounded when diseases with similar or interacting symptoms are found in the same range (Goodwin, 2007) (e.g. Septoria can be difficult to distinguish from other leaf blights or from yellow and brown rusts).

Unfortunately, some phenotyping is still unavoidable in breeding programs when the effectiveness of new resistances is first being analysed, or during gene mapping efforts such as those targeted to narrow down the genetic region where a resistance gene may be located. Therefore, effort has been put into the development of modern imaging and analysis techniques that can provide more objective data with a level of detail that it would not otherwise be possible to acquire in the same timeframe (Karisto *et al.*, 2017; Karisto *et al.*, 2019). Confocal microscopy studies focusing on stomatal penetration by fluorescent *Z. tritici* isolates have added additional information to this, helping to elucidate potential mechanisms that may help to explain

much of the resistance of wheat lines protected by *Stb* genes including *Stb16q* (Battache *et al.*, 2022).

In the analysis of *Z. tritici* infections, these modern imaging and analysis techniques are likely to be most helpful in the counting of pycnidia on diseased leaves and the assessment of pycnidia size (Karisto *et al.*, 2017). This is extremely difficult to do by eye alone and is not usually attempted, with generalised one to five scales being the preferred method of most researchers for quantifying leaf coverage by pycnidia. However, machine learning can be used to train imaging software to identify individual pycnidia in high quality scans of leaf surface images, allowing the pycnidia present to be counted and sized automatically. This could give very precise quantifications of infection levels that could be very useful in identifying new disease resistance loci, especially those controlling partial resistances.

Other imaging methods for improving the efficiency of phenotyping have also been developed. For example, changes to the fluorescence of the leaf cells have been identified as among the first disease symptoms to become detectable during the early stages of fungal infection due to changes in plant photosynthesis patterns (Mutka and Bart, 2015; Mihailova *et al.*, 2019). It may therefore be possible to measure leaf fluorescence to provide more information on the onset rates of fungal infections, and to predict the severity of symptoms such as leaf necrosis (Spyroglou *et al.*, 2022). Other work has indicated that membrane integrity (potential estimated from lipid compositions, such as peroxidation levels) and electron leakage could be important early indicators of resistance levels in wheat during *Z. tritici* infection (Mihailova *et al.*, 2019).

Another alternative method of phenotyping is using drones and other aerial photography technology, together with networks capable of automated analysis of the large amounts of data this would produce (Deng *et al.*, 2022). This allows data to be collected very quickly at the field and plot level during larger scale trials. The most useful application of this technology in disease resistance studies is likely to be using infrared cameras to track transpiration rates from plant leaves by monitoring temperature changes relative to ambient conditions, which will provide information about stomatal opening and leaf senescence (Wang *et al.*, 2019). These techniques are likely to be best suited to the study of the spread of infection.

In addition to improvements in our ability to genotype and phenotype plants, further development must involve improvements to the processes used to inoculate test plants. Currently, the inoculum used in experiments relating to Septoria resistance usually comes from two sources – natural infection from nearby farms (used when plants are grown to adulthood under field conditions in breeding work) and

laboratory strains. This can lead to issues with identifying effective resistances, as often results from these two types of infection do not match well. Many wheat lines identified as possessing field resistance to Septoria appear to be susceptible to individual fungal isolates in lab inoculations (possibly due to the high levels of inoculum generally applied in lab studies, which often exceeds any exposure likely to occur in the field). Meanwhile, many of the laboratory *Z. tritici* isolates often used in laboratory experiments originate from old field populations and are still naïve to many resistances and fungicides which are no longer effective against modern field populations (e.g. IPO323, the most common reference strain, is naïve to the widespread and widely broken resistance gene *Stb6* (Chartrain, Brading, *et al.*, 2005)).

These issues are compounded by the fact that most lab studies of *Stb* gene effectiveness only test the wheat lines carrying these genes against a very limited selection of *Z. tritici* isolates (for example, 22 isolates were used in Cowger *et al.* (2000), ten in Dalvand *et al.* (2018), and only one in Ali *et al.* (2008)). This is not a good representation of field populations as due to its ability to reproduce sexually, *Z. tritici* can maintain great population diversity even in small areas (Mekonnen *et al.*, 2020). These issues can make it difficult to translate the available literature into good predictions of the field effectiveness of a given *Stb* gene, an issue compounded by the different disease scoring scales and methodologies used for analysing infection phenotypes (Stewart *et al.*, 2016; Karisto *et al.*, 2017; Karisto *et al.*, 2019).

Solving these issues would provide us with a better understanding of the effectiveness of each *Stb* gene under field conditions, as well as improving our ability to identify resistance QTLs and assess their contributions to resistance effectively. This in turn could lead to the development of new diagnostic genetic marker sets that could be applied during the seedling stage in breeding programs to identify lines with acceptable Septoria resistance levels early.

This process will likely be further aided by the application of modern gene editing and manipulation techniques such as CRISPR/Cas9, which will facilitate rapid reverse genetics and complementation studies to identify important genes on both the plant and fungal side of the interaction (e.g. *Stb* genes and the matching fungal avirulence genes). This technique has already lead to important advances for similar plant pathosystems (e.g. knocking out the RXLR effector gene *Avr4/6* in *Phytophthora sojae* to establish its contribution to the recognition of the pathogen by the soybean resistance genes *Rps4* and *Rps6* (Fang and Tyler, 2016)).

Such fundamental research will be further facilitated by the development and application of new tools, such as Virus Induced Gene Silencing (VIGS), for which specialised protocols have been developed

to optimise the process for use in wheat in conjunction with *Z. tritici* inoculation (W.,-S., Lee *et al.*, 2015). This will allow candidate disease resistance genes and susceptibility factors to be easily knocked down for investigations of their functions, providing data on the molecular interactions necessary for *Z. tritici* to successfully infect wheat and thus potentially identifying resistance genes and new potential targets for future fungicides. This process will be simplified by the recent development of methods allowing *Agrobacterium tumefaciens* to deliver *Barley stripe mosaic virus* (used to modify plant genes since the early 2000's, now a common VIGS vector) to plant cells (Yuan *et al.*, 2011). VIGS has been utilised to examine disease resistance genes in wheat pathosystems (e.g. the role of the *SnRK1α* gene family in *Fusarium* toxin tolerance (Perochon *et al.*, 2019) or the characterisation of wheat mitochondrial phosphate transporter (TaMPT)- and methyltransferase (TaSAM)-encoding wheat genes involved in Fusarium head blight resistance (Malla *et al.*, 2021)), and could enable similar rapid breakthroughs in research for Septoria resistance.

8. Future prospects and long-term research goals

To achieve the long-term goal of durable Septoria resistance in elite wheat cultivars, we must develop a system in which the currently available *Stb* genes and resistance QTLs can remain effective long enough for more durable background resistances to be bred in. For other wheat diseases such as rusts and mildews this process has required decades of effort to come to completion, suggesting that to protect our crops until this point we may have to seriously increase the diversity and effective duration of the *Stb* genes we use. Gene pyramiding currently seems the most viable solution, but this is likely to require the development of modern genetic markers for any *Stb* resistance genes for which these are not already available, as the already challenging task of following resistances through a breeding population by phenotyping alone will become almost impossible when attempting to track and differentiate between multiple resistances in the same population.

These markers will be more effective if positioned closer to, or ideally within, the genes of interest. The optimal method for accomplishing this would be through cloning of the resistance genes and development of markers targeting the specific differences between susceptible and resistant varieties. However, the cloning of such genes would require significant time and expense in each case. It is therefore likely that many breeders will focus on the production and testing of markers close to the expected sites of these resistance genes and rely on genetic linkage with these markers to follow resistances in breeding populations, which will require considerably less work. Fortunately,

these markers can still be used by the scientific community to narrow down the potential genetic locations of *Stb* resistance genes in larger studies.

Cloning of resistance genes of interest will support further functional analysis of *Stb* genes by allowing us to analyse the amino acid sequences of the proteins they encode and homology to other genes, as well as enabling over and under expression studies and allowing the associated proteins to be produced in other (heterologous) systems for further functional study if necessary. Given the importance of Septoria in European agriculture, even high investment studies such as X-ray crystallography of the isolated proteins may be justifiable to funding bodies, providing far greater insight into the functioning of this system than we currently possess. This specific example assumes that the resistance related proteins can be crystallised correctly and that such studies would provide useful information to the scientific community – these assumptions may be challenged by the nature of the cloned genes (for example, membrane spanning proteins such as wall associated kinases may be extremely difficult to crystallise correctly), potentially making simpler systems more suitable for their examination. For example, simply affixing resistance proteins to a plate or membrane, washing fungal extracts over them and analysing those that bind could help to determine both fungal avirulence protein identity and resistance binding mechanisms through co-immunoprecipitation (Gawehns *et al.*, 2013). Such interactions can be confirmed with yeast two hybrid screens or bimolecular fluorescence complementation (Janik *et al.*, 2019; Pelgrom *et al.*, 2020).

Such insights into the mechanics of this pathosystem could enable us to uncover commonalities between the different *Stb* resistance genes. For example, it is possible that many of them, similarly to *Stb6* (Saintenac et al. 2018) and the Fusarium head blight resistance QTL *QFhb.mgb-2A* (Gadaleta *et al.*, 2019) encode wall-associated receptor-like kinase (WAK) proteins, or that, similarly to *Stb16q,* many other *Stb* genes encode plasma membrane cysteine-rich receptor-like kinases (Saintenac *et al.*, 2021). Understanding such commonalities could help target future disease resistance gene identification efforts in forward genetic screens or allow the identification of new resistance genes in reverse genetics efforts (particularly in the less well studied landraces, ancestor species and synthetic wheat lines). It is also possible that common patterns will be identified between more or less durable *Stb* genes (e.g. perhaps durable genes interact with certain essential fungal effectors), which could help to predict the durability of newly discovered resistance genes and plan breeding strategies accordingly.

Such understanding of host defence can also be aided by research into broader resistance mechanisms activated by the resistance receptors and messenger proteins (e.g. stomatal closure or hypersensitive

responses). Improved near-isogenic lines (NILs) produced using improved resistance markers will enable the examination of such resistance mechanisms for a wider variety of *Stb* resistance genes, while a suitable database of *Z. tritici* isolates that records their virulence levels against different *Stb* resistance genes could help to more easily identify a number of isolates suitable for use in such testing. It would be further beneficial if some of these *Z. tritici* isolates (i.e. a selection of those with highest virulence and known avirulence against *Stb* resistance genes of interest) could be marked (e.g. using fluorophores) to make the measurement and analysis of their infection patterns more convenient.

The development of improved methods for stable genetic transformation of *Z. tritici* isolates has opened productive new avenues for conducting this kind of research into the mechanisms of host resistance genes. For example, genetically modified *Z. tritici* strains expressing fluorescent reporter proteins have allowed recent analyses to identify the *Stb16q*-mediated resistance appears to operate at the stomatal level (with the resistance response leading to stomatal closure to prevent fungal entrance into the leaf) through the use of confocal microscopy (Battache *et al.*, 2022). This seems likely to indicate that initial effector recognition in this pathosystem occurs before leaf penetration, and is possibly carried out by surface receptors in host guard cells. This indicates that the leaf surface may be a more important site for *Z. tritici* recognition then was initially assumed – such basic information is valuable when attempting to pyramid resistance genes to form an effective durable resistance.

Plant defences (especially those activated by leaf surface receptors) could also be reinforced by the development of new defence-priming compounds such as tramesan, a polysaccharide secreted by the Turkey tail fungus *Trametes versicolor* that seems able to activate wheat immune defences and reduce reactive oxygen species levels, thus increasing resistance to *Z. tritici* (Scala *et al.*, 2020). As this compound seems to affect the plant directly, it is unlikely that fungal pathogens could develop significant resistance to its effects, but such defence activating compounds will only be effective as long as some level of host resistance exists in the wheat lines they are used on. Further testing may be necessary to examine the effects of such compounds on wheat resistance to other crop pathogens, as wheat immune responses to biotrophic and necrotrophic pathogens can be antagonistic, and the value of such protective compounds would be greatly reduced if they cause increased susceptibility to other pathogens.

9. Thesis hypothesis and aims

In order to contribute to these research needs, three primary sections of this project were devised. The first of these was a large bioassay screen, in which large numbers of 2015-2017 UK *Z. tritici* isolates were tested against wheat genotypes carrying a total of 17 known *Stb* resistance genes. The aim was to produce a dataset showing which *Z. tritici* isolate were virulent and which were avirulent against each *Stb* resistance gene, from which the breadth of each tested resistance against UK *Z. tritici* populations could also be compared.

Hypotheses: That *Stb* resistance genes would vary in the average levels of resistance they provided to UK *Z. tritici* isolates, and in which specific *Z. tritici* isolates they provided resistance to.

Aims: To identify *Stb* resistance genes that remain broadly effective against an array of UK *Z. tritici* isolates, and potential resistance gene pyramids that could provide resistance to all UK *Z. tritici* isolates in our library. Additionally, to produce a database of UK *Z. tritici* isolates with estimated levels of aggressiveness for a wide array of isolates that could be used to identify likely suitable isolates for future work involving wheat inoculation with *Z. tritici* isolates under laboratory conditions.

The second section of this project used backcrossed segregating and quasi-NIL populations produced from the susceptible KWS Cashel and wheat lines containing *Stb* resistance genes of interest, generated from three generations of selfing or backcrossing, respectively. The segregating populations were used in the testing and verification of KASP markers designed to co-segregate with *Stb* genes of interest, while the NIL lines were used to differentiate between the contributions of resistances for which the relevant resistant line contained multiple resistant genes. The primary aims were to identify a series of KASP markers that co-segregate with resistance genes of interest, and to determine the contribution of each *Stb* resistance gene to the total resistance of wheat lines such as Synthetic M3 and Kavkaz-K4500, which have multiple such genes.

Hypotheses: That KASP markers based on SNPs close to the expected genomic locations of known resistance genes can function to follow *Stb* resistance genes in a segregating population. Also, that *Stb* resistance genes of interest would continue to provide host plants with protection from STB in a predominantly KWS Cashel background, and that where multiple *Stb* resistance genes were present in a single wheat genotype some of these *Stb* resistance genes would contribute broader protection against UK *Z. tritici* field isolates than others.

Aims: To produce verified KASP markers for each of the primary resistance genes of interests identified in the previous chapter which can be used by breeders to more easily introduce the associated

resistance into their lines, as well as confirming the heritability of the resistance traits associated with these *Stb* resistance genes. Additionally, this chapter aimed to further elucidate the effectiveness of resistance genes that may have been concealed by the epistatic effects of other resistance genes in the previous chapter and to confirm that this resistance remained effective with alternative genetic backgrounds using NIL lines containing *Stb* resistance genes of interest.

The final research section of this project aimed to produce and test fluorescent *Z. tritici* strains as research tools to enable the investigation of host defence mechanisms associated with different *Stb* resistance genes.

Hypothesis: That the fluorophores GFP and mCherry can be transformed into *Z. tritici* isolates identified as having virulence profiles useful for the examination of *Stb* resistance gene mechanisms in previous chapters, and that this would produce *Z. tritici* strains with known virulence profiles that could be visualised under a confocal microscope. Additionally, it was hypothesised that the *Stb* resistance gene *Stb5* operated at least partially through a mechanism of stomatal closure in response to leaf surface recognition of avirulent *Z. tritici* strains.

Aims: To produce fluorescent *Z. tritici* strains transformed with the fluorophores GFP and mCherry, to be used in further research, and to test the virulence of theirs strains and confirm that they could be visualised correctly. Additionally, to use these fluorescent strains to determine the extent to which stomatal closure is relevant to the resistance mechanism activated by the *Stb5* resistance gene through confocal microscopic comparison of compatible and incompatible host-pathogen interactions.

It is hoped that the work described in this thesis will provide both breeders and researchers with new tools and data that can be used in the production of superior wheat lines and the construction of further experiments to deepen our understanding of this pathosystem. Such tools and knowledge will be important in ensuring that appropriately broadly effective resistances can be identified and pyramided in future wheat varieties to provide broad, durable resistance. The procedures optimized in this thesis could also be used to carry out similar work for worldwide *Z. tritici* populations in order to help identify optimal *Stb* resistance gene combinations for breeders around the world.

Chapter 2 - A large bioassay identifies *Stb* resistance genes that provide broad resistance against Septoria tritici blotch disease in the UK

Previously published as Tidd *et al.*, 2023, with minor modifications.

Abstract

Introduction: Septoria tritici blotch (STB) is one of the most damaging fungal diseases of wheat in Europe, largely due to the paucity of effective resistance genes against it in breeding materials. Currently dominant protection methods against this disease, e.g. fungicides and the disease resistance genes already deployed, are losing their effectiveness. Therefore, it is vital that other available disease resistance sources are identified, understood and deployed in a manner that maximises their effectiveness and durability.

Methods: In this study, we assessed wheat genotypes containing nineteen known major STB resistance genes (*Stb1* through to *Stb19*) or combinations thereof against a broad panel of 93 UK Zymoseptoria tritici isolates. Seedlings were inoculated using a cotton swab and monitored for four weeks. Four infection-related phenotypic traits were visually assessed. These were the days post infection to the development of first symptoms and pycnidia, percentage coverage of the infected leaf area with chlorosis/necrosis and percentage coverage of the infected leaf area with pycnidia.

Results: The different *Stb* genes were found to vary greatly in the levels of protection they provided, with pycnidia coverage at four weeks differing significantly from susceptible controls for every tested genotype. Most lines were found to show considerable variation in their symptom levels when inoculated with different *Z. tritici* isolates, with only the most resistant or susceptible lines showing reliable symptoms when challenged with a range of pathogen isolates. *Stb10*, *Stb11*, *Stb12*, *Stb16q*, *Stb17*, and *Stb19* were identified as contributing broad spectrum disease resistance (with no single *Z. tritici* isolate identified as possessing virulence against all 6 *Stb* resistance genes), and synthetic hexaploid wheat lines were identified as particularly promising sources of broadly effective STB resistances. No evidence was found to indicate that the wheat line from which a *Z. tritici* isolate was originally isolated could be used to predict the overall aggressiveness of that isolate.

Discussion: Wheat genotypes carrying multiple *Stb* genes were found to provide higher levels of resistance than expected given their historical levels of use. In some cases, it was possible to largely discount the effects of some resistance genes in such lines as significant contributors to the total resistance, due to the lack of effectiveness that genes such as *Stb6* and *Stb7* displayed in other wheat lines that possessed fewer resistance genes. Furthermore, it was noted that disease resistance controlled by different *Stb* genes was associated with different levels of chlorosis, with high levels of early chlorosis in some genotypes correlated with high resistance to fungal pycnidia development. This may suggest the presence of multiple resistance mechanisms. The knowledge obtained here will aid UK breeders in prioritising *Stb* genes for future breeding programmes,

in which optimal combinations of resistance genes could be pyramided. In addition, this study identified the most interesting *Stb* genes for cloning and detailed functional analysis.

Contributions

My contributions to this chapter include:

- The growth, maintenance and where necessary purification of all *Z. tritici* isolates used.
- The growth and preparation of all host plants used.
- The inoculation of host plant leaves.
- The phenotyping of inoculated plants throughout symptom development.
- The collation, statistical analyses and interpretation of results.
- The initial drafting of this work as a scientific paper, updating drafts in response to advice from my supervisory team and journal reviewers and submitting this paper through the Frontiers in Plant Sciences process.

The contributions of others to this chapter include:

- The initial provision of fungal isolates used by Bart Fraaije.
- Advice from my supervisory team (Kostya Kanyuka, Jason Rudd, Rumiana Ray and Ruth Bryant) throughout the experimental and writing processes.
- Advice from Frontiers in Plant Sciences reviewers Steven Kildea and Lise Nistrup Jørgensen during the final preparation of this manuscript.

Introduction

Septoria tritici blotch (STB), caused by the fungal pathogen *Zymoseptoria tritici*, is one of the most damaging wheat diseases across Europe, with the capacity to cause up to 50% crop losses under disease-favourable conditions (Fones and Gurr, 2015). Approximately 70% of the fungicides used in Europe can be for the purpose of preventing *Z. tritici* epidemics (Duveiller *et al.*, 2007; Torriani *et al.*, 2015). Developing methods for protecting wheat from STB is therefore a high priority for UK wheat breeders and researchers.

Traditionally, STB protection has been achieved through the widespread application of fungicides reinforced with the deployment of a small number of *Stb* resistance genes. However, the sexual reproductive cycle that *Z. tritici* undergoes around the end of the breeding season can contribute to high levels of genetic diversity in the pathogen, leading to the rapid loss of effectiveness from fungicides. A similar lack of durability has proven an issue with *Stb* resistance genes. For example, *Stb6* and *Stb15* have both been widely used in Northern Europe and were initially highly effective however, both have since

been widely broken by *Z. tritici* due to the selection pressures caused by their widespread use (Arraiano *et al.*, 2009; Stephens *et al.*, 2021). *Stb16q* has also been brought into wide use more recently in some European countries, and initially offered very broad STB resistance. However, virulent isolates of *Z. tritici* against *Stb16q* have already been reported in Iran, Ireland and France (Dalvand *et al.*, 2018; Kildea *et al.*, 2020; Orellana-Torrejon *et al.*, 2022) and are likely to spread rapidly within field populations, making this resistance gene less useful in future breeding programmes. The lack of broad spectrum STB resistance in wheat leaves agricultural systems vulnerable when major resistance genes are broken (e.g. the cultivar Gene in the USA, which was fully resistant in 1992 but widely susceptible by 1995, causing substantial crop losses (Cowger *et al.*, 2000), or Cougar, which has become unpopular with growers due to the development of Cougar-virulent strains of *Z. tritici* in the UK (Kildea *et al.*, 2021)). Such problems will only become more frequent as effective fungicide protection options become more limited (Birr *et al.*, 2021).

It is also noteworthy that some individual major resistance genes that have been widely used in breeding so far have proved to be more durable than others. For example, *Stb1* was introduced to the grower market in the cultivar Oasis in 1975 and has been used in many other cultivars (e.g. Sullivan) since 1979 and remained effective in the field up until mid-2000's (Cowger *et al.*, 2000; Adhikari *et al.*, 2004; Singh *et al.*, 2016). *Stb4* also proved to be reasonably durable, lasting for approximately 15 years. After its introduction to breeding programs in 1975 (in a cross between Tadorna, Cleo and Inia 66), the first cultivar containing *Stb4* underwent a commercial release in 1984 (Somasco *et al.*, 1996), and this gene remained effective until 2000 (Jackson *et al.*, 2000). However, no individual *Stb* gene so far identified appears to be completely durable. Gene pyramiding may be able to mitigate this rapid breaking of disease resistance by producing additional obstacles to fungal populations in the evolution of new virulences. For example, Kavkaz-K4500 is one of the most durable sources of field resistance used for breeding and has been shown to possess at least five qualitative resistance genes, including *Stb6*, *Stb10* and *Stb12* (Chartrain, Berry, *et al.*, 2005). This combination of *Stb* genes seems to be sufficient to make Kavkaz-K4500 resistant to STB under field conditions despite the fact that many international *Z. tritici* isolates are virulent on it in laboratory tests (Chartrain *et al.*, 2004a; Chartrain *et al.*, 2005a) – this may suggest high genetic diversity differences between UK and international *Z. tritici* populations, or could be related to the different levels of inoculum used in laboratory vs field trials.

The currently limited availability of data on the interaction between modern *Z. tritici* isolates and wheat, along with the difficulty in comparing data from different sources, is problematic as it has limited

our ability to identify useful sources of quantitative resistances to this disease (Chartrain *et al.*, 2004). This combined with the limited historical breeding for STB resistance, has led to a dearth of cultivars with significant quantitative resistance to the disease.

Further issues arise from the lack of standardised, modern wild-type *Z. tritici* isolates among the standard model strains for this disease, which represents a significant obstacle to the development of durable STB resistance in wheat. This is due to the difficulties it causes in designing experiments that produce information on the likely field efficacy of resistance genes and quantitative trait loci (QTLs) that can be easily compared to other work in the same field. It is therefore important that new field isolates of *Z. tritici* are collected from all regions of interest for breeders, to be used in the testing of new resistance genes. A database of *Z. tritici* isolates with known virulence profiles could help identify combinations of *Stb* resistance genes that could provide several independent resistances for each tested *Z. tritici* isolate. This could allow the identification of combinations of resistance genes that would require several independent mutations in any *Z. tritici* isolate in order for that isolate to gain virulence.

This rapid breakdown of existing resistances makes it particularly important that breeders have access to novel STB resistance genes effective against local *Z. tritici* populations. Several known major resistance genes, such as *Stb5*, *Stb17* and *Stb19*, have not previously been widely used in Europe, and could perhaps be used to replace those that have already been overcome (e.g. *Stb6* and *Stb16q*). Unfortunately, little data is currently available to breeders regarding which of these genes are sufficiently broadly effective to be worth using in breeding programs.

It is therefore clear that a future priority in wheat breeding is likely to be the development of elite lines containing a greater variety of disease resistance genes. Major resistance genes are likely to be a large part of this as they can be identified easily and applied quickly in breeding programs, and major genes not yet broken will provide excellent field resistance. More than twenty *Stb* resistance genes that could be used in wheat breeding programs have thus far been identified, providing natural protection against a variety of *Z. tritici* isolates at the different stages of the wheat life cycle (referred to as seedling and adult resistance genes) (Dreisigacker *et al.*, 2015). For many of these *Stb* genes we have some information relating to their chromosomal locations, but in the majority of cases this data is imprecise.

Overall, large pathology screens are necessary to assess the effectiveness of *Stb* genes more accurately. Conducting these screens on more genetically diverse germplasm (particularly non-elite landraces and ancestor species) may help to identify novel *Stb* genes highly

effective against current *Z. tritici* populations. Here we carried out a broad screen of 2015-2017 UK *Z. tritici* isolates against a panel of wheat lines of diverse origin containing known *Stb* resistance genes to produce estimates of the effectiveness of each of these genes against contemporary field populations of *Z. tritici* in the UK.

The primary hypotheses of this research were: (1) That there would be significant differences in the breadth of the resistance offered by different known major *Stb* resistance genes with respect to the diversity present in UK *Z. tritici* isolates, and (2) that it would be possible to identify a subset of broadly effective *Stb* resistance genes that would provide good resistance against all UK *Z. tritici* isolates in a broad panel.

Through this work, several *Stb* genes were identified as contributing broad spectrum disease *Stb* resistance in wheat, and synthetic hexaploid wheat lines were identified as promising sources of broadly effective STB resistance.

Materials and methods

Library of fungal isolates

One hundred *Z. tritici* isolates were donated by Bart Fraaije (NIAB, UK). These isolates were collated from locations around the UK in the years 2015-2017. These isolates were originally drawn from many sources with different naming conventions, and were renamed for ease of use in this project – a list of the original names of these isolates on receipt is included in the Supplementary data.

In preparation for use in these experiments, these isolates were grown on 7% (w/v) YPD agar (Formedium Ltd., Hunstanton, UK) plates containing 1 unit of penicillin and 1 µg/mL streptomycin (Merck Life Science UK Limited, Gillingham, UK) to remove bacterial contamination. Approximately 25 µl of original *Z. tritici* glycerol stocks were used per plate. Inoculated plates were incubated at 16ºC for four to seven days before the fungus was harvested using a sterile loop into 50% (w/v) glycerol and stored at -80ºC. This was then repeated using antibiotic free YPD plates to ensure the fungi used were not stressed. Fungi from antibiotics-free plates were harvested and stored identically. Large amounts for fungal material was harvested in the initial harvesting in order to ensure that additional bulking up of fungal material was not generally necessary during this PhD (with limited exceptions for isolates afflicted with fungal or bacterial contamination, which were re-plated twice). This was expected to prevent any genetic drift in the fungal isolates used during the experiments by preventing the passage of sufficient generations to enable this issue to arise. Once pure stocks had been produced, extracts from the same stock

(stored consistently at -80°C to ensure that it does not deteriorate) were plated as needed to grow new inoculum for each isolate.

Where bacterial contaminants proved resistant to the antibiotics used, contaminated glycerol stock was diluted (approximately by a factor of 100, depending on concentration), allowing individual colonies to form from single spores or cells. Suitable uncontaminated *Z. tritici* colonies were harvested into 50% glycerol and re-plated to produce pure stocks. This required stocks to be re-plated twice, once during the dilution step to allow selection of a pure colony and once more to allow harvesting of the purified fungal material. Large amounts of material where then harvested and stored as before to minimise the need for re-plating and the opportunity for genetic drift.

Wheat lines used

Wheat lines were chosen for use in this study that collectively contained *Stb* resistance genes *Stb1*-*Stb19*. These lines and the *Stb* genes they contain are listed in Table 1. Taichung 29 and KWS Cashel were both included as known susceptible controls (of these, KWS Cashel was the primary control and Taichung 29 was included as a second control in case KWS Cashel was found to be resistant to any *Z. tritici* isolates used). This wheat seed was primarily sourced from existing stocks maintained at Rothamsted Research, with additional seed provided by RAGT Seeds Ltd. Seed was stored in dark, room temperature conditions in sealed Falcon tubes until required, then prepared for use by immersion in water for 24 hours (also under dark, room temperature conditions in sealed Falcon tubes) before planting. Despite long-term storage, most of the wheat seed used showed good germination rates throughout the PhD project, and was used in the work described through all following experimental chapters.

Inoculation of wheat plants

Z. tritici isolates used in inoculations were cultured on antibiotic-free YPD agar plates and grown for four to seven days at 16ºC. Fungal blastospores were then harvested using sterile loops into 5mL of 0.1% Silwet L-77 surfactant (Momentive Performance Materials, Waterford, NY, USA) in H_2O and diluted to a concentration of 10^7 spores per mL using the average of two replicated measurements from a haemocytometer. High concentrations and the presence of a surfactant are not reflective of field conditions but were included to encourage rapid infection to reduce the time needed per bioassay.

Plants were grown for approximately three weeks (adapted for variable growth rates where necessary) at 16-hour day, 8-hour night cycles under halogen or white LED lamps at a temperature of 21°C and ambient humidity. After inoculation, these plants were transferred to 17°C and the same 16-hour day, 8-hour night cycle. The second leaf was inoculated where possible, although for some cultivars (e.g. Israel

493) the third leaves were used due to their larger size. One leaf each from a minimum of three plants was used for testing each wheat genotype - *Z. tritici* isolate interaction.

Leaves were affixed to aluminium inoculation tables using double sided sticky tape and rubber bands, which also defined the area inoculated and scored. Cotton buds were used to inoculate each spore suspension onto leaves of three plants of each wheat line (four strokes per leaf, ensuring an even layer of moisture on leaf surface). Non-inoculated leaves were trimmed to ensure light access to inoculated leaves.

After inoculation, plants were placed in high humidity boxes (Supplementary Figure 1) for three days before the inner tray (perforated to allow for water uptake) was removed and placed in a larger plastic watering tray to minimise the risk of causing leaf damage or cross-contamination from direct watering.

Plants were maintained for 28 days after inoculation to allow symptom development. They were watered three times per week and kept trimmed to ensure light access to inoculated leaves. From ten days post inoculation (dpi), plants were checked regularly (every two days where possible) for chlorosis, necrosis and pycnidia development, and symptoms were recorded. Photographs were taken at each check for later verification.

The final screen included 973 tested interactions. Due to the large number of wheat genotype – *Z. tritici* isolate interactions tested, one replicate was normally performed for each of these interactions in the bioassay.

Visual symptom assessments

Necrosis, chlorosis and pycnidia development symptoms were assessed visually. Assessment of the rate of symptom and pycnidia development began ten days after seedling inoculation by *Z. tritici* for each plant. Assessments were then carried out three times a week at regular intervals until 28 days after the initial inoculation date. Leaf status was recorded as no infection (i.e. clean), chlorosis present (showing yellow chlorotic tissue but which had not yet progressed to necrosis), necrosis present (where necrotic lesions were visible), chlorosis with pycnidia (chlorotic symptoms present with small black pycnidia visible on the inoculated leaf surface) or necrosis with pycnidia. The first date on which chlorosis or necrosis was seen was used to determine the “days until symptom development” trait value, while the date on which pycnidia were first noted was used to determine the “days until pycnidia development” trait value. Photographs were taken at each check in case needed for later verification of results.

Table 1: Wheat lines used in this study with known *Stb* genes.

Wheat Genotype	Known *Stb* genes	Reference
Taichung 29	No *Stb* genes known	-
KWS Cashel	No *Stb* genes known	-
Bulgaria 88	*Stb1, Stb6*	Adhikari *et al.*, 2004
Veranopolis	*Stb2, Stb6*	Liu *et al.*, 2013
Israel 493	*Stb3, Stb6*	Goodwin *et al.*, 2015
Tadinia	*Stb4, Stb6*	Adhikari *et al.*, 2004
Synthetic 6X	*Stb5*	Arraiano *et al.*, 2001
Estanzuela Federal	*Stb7*	McCartney *et al.*, 2003
Synthetic M6 (Previously W7984)	*Stb8*	Adhikari *et al.*, 2003
Tonic	*Stb9*	Chartrain *et al.*, 2009
Kavkaz-K4500	*Stb6, Stb7, Stb10, Stb12*	Chartrain, Berry, *et al.*, 2005
TE9111	*Stb6, Stb7, Stb11*	Chartrain, Joaquim, *et al.*, 2005
Salamouni	*Stb6, Stb13, Stb14*	Cowling, 2006
Riband	*Stb15*	Arraiano *et al.*, 2007
Synthetic M3	*Stb16q, Stb17*	Ghaffary *et al.*, 2012
Balance	*(Stb6), Stb18*	Ghaffary *et al.*, 2011
Lorikeet	*Stb6, Stb19*	Yang *et al.*, 2018

At 28 days post infection, before leaves were harvested, the "percentage leaf area covered by symptoms" and "percentage leaf area covered by pycnidia" traits were visually assessed. The values for each leaf were rounded to 0, 20, 40, 60, 80 or 100% for each leaf. Photographs were taken in case needed for later verification of results.

Statistical analysis

Statistical tests were carried out using the statistics package R (R Core Team, 2017) to run paired Student's *t*-tests on data from different wheat lines (results obtained using the same *Z. tritici* isolate in the same experimental set were treated as paired) using standard R commands for this function. The large numbers of *Z. tritici* isolates tested against the wheat genotypes of interest allowed for statistical assessments of the average broad resistance of each line. ANOVA tests were used when data from multiple wheat lines was to be compared, and to verify results produced from the *t*-tests – this was done using standard R and Excel Data Analysis commands.

Results

The assessment of multiple phenotypic traits for a large panel of *Z. tritici* isolate – wheat genotype interactions

Seventeen wheat genotypes carrying no known *Stb* gene, a single *Stb* gene, or a combination of *Stb* genes were screened against up to 100 current UK *Z. tritici* isolates. The symptoms of each genotype were compared to those of KWS Cashel, used as the susceptible control. The *P*-values derived using a standard student's t-test to compare the average % pycnidia coverage of inoculated leaf area for each *Z. tritici* isolate-resistant wheat line to the equivalent averages from interactions with the KWS Cashel susceptible control are shown in Table 2, along with the average values for this phenotypic measurement in each line – these data show which lines have significantly different symptom development levels overall than KWS Cashel ($P<0.05$), and suggest that KWS Cashel is significantly more susceptible than any other tested wheat line. Mean average values the full set of genotype-isolate comparisons tested on each wheat line are given in Table 3 for each of the four measured traits. The proportion of isolate-wheat line interactions for which disease symptoms were entirely absent for chlorosis/necrosis and for pycnidia development is shown in Table 4.

Inoculated wheat plants were assessed for four STB disease associated symptoms: the times (dpi) taken for the initial developments of chlorosis/necrosis symptoms and fungal pycnidia, the final percentage of the inoculated leaf sections covered by chlorosis/necrosis and the final percentage of the inoculated leaf sections covered by pycnidia. Attempts were also made to quantify

fungal sporulation in the inoculated leaves at 28 days post inoculation using spectrophotometry, but the obtained data was considered unreliable due to systemic over-counting of spores by this method and was thus omitted for clarity.

Trait 1: Time to appearance of first symptoms

The time to the appearance of symptom development on each seedling was measured as the number of days taken from inoculation to the first visible chlorosis or necrosis on the inoculated leaf area. There was significant biological variation in the rates of development of chlorosis and necrosis symptoms and percentage of leaf coverage by chlorosis/necrosis in some wheat line – *Z. tritici* isolate interactions (potentially caused by variation in factors such as sunlight levels, natural senescence or mechanical damage done during inoculation). This trait is therefore considered the least reliable indicator of virulence presented here. The wheat genotype that showed chlorosis/necrosis symptoms soonest on average was Taichung 29 at just 13 days post inoculation (dpi), although Israel 493 and Estanzuela Federal were close to this (13.6 and 14.1 dpi, respectively). The slowest average development of infection symptoms was in Lorikeet, with an average of 18.9 dpi.

Trait 2: Time to appearance of first pycnidia

The time to the appearance of pycnidia for each seedling was measured as the number of days taken from inoculation to the first visible pycnidia on the inoculated leaf area. The lowest average time to the appearance of pycnidia was 16.4 dpi in the wheat genotype Taichung 29. This value could not be obtained for Synthetic M3, Kavkaz-K4500 or Lorikeet due to the complete lack of pycnidia development in these genotypes. It should be noted that as this trait was not usually measurable in incompatible (resistance) interactions, the values provided for this apply only to interactions that enabled some level of pycnidia formation.

Trait 3: Inoculated leaf coverage by symptoms

The final percentage of the inoculated area of the leaf covered by chlorosis and necrosis at 28 days post inoculation was expected to provide an estimate of the relative levels of photosynthetic loss that could be expected from each wheat genotype when challenged with each isolate of *Z. tritici*. This trait showed high levels of variation both within and between wheat genotypes (Figure 1). Only highly resistant or highly susceptible genotypes showed more restricted ranges, with Estanzuela Federal leaves having consistently high symptoms coverage and leaves of Kavkaz-K4500 displaying consistently lower symptoms coverage. Due to these high ranges in the results obtained from most genotypes and the potential for occasional leaf damage due

Table 2: A comparison of the % pycnidia coverage of inoculated leaf area for each *Z. tritici* isolate-resistant wheat genotype interaction and the equivalent values derived from the *Z. tritici* isolate's interactions with the KWS Cashel susceptible control. The average pycnidia coverage of KWS Cashel leaves can be seen to be significantly higher than that of all other tested wheat lines.

Wheat Genotype	Average final % of inoculated leaf area covered by pycnidia	*P*-value
KWS Cashel (Susceptible control)	**36**	**-**
Taichung 29	**21**	**1.5×10^{-5}**
Riband	**23**	**3×10^{-4}**
Synthetic 6X	**1**	**4.6×10^{-12}**
Synthetic M3	**0**	**1.6×10^{-10}**
Kavkaz-K4500	**0**	**4×10^{-13}**
Tadinia	**9**	**7.3×10^{-8}**
Estanzuela Federal	**10**	**1×10^{-10}**
Israel 493	**1**	**7.2×10^{-16}**
TE9111	**1**	**5.8×10^{-18}**
Bulgaria 88	**3**	**2.1×10^{-6}**
Veranopolis	**6**	**1.9×10^{-6}**
Synthetic M6	**10**	**4.4×10^{-5}**
Tonic	**15**	**1.3×10^{-2}**
Salamouni	**3**	**3×10^{-4}**
Balance	**3**	**2.3×10^{-6}**
Lorikeet	**0**	**5.9×10^{-7}**

A mean average from each interaction (calculated using the standard function in excel) were compared to that with KWS Cashel using a two-tailed Student's *t*-test from the excel data analysis tool. The *P*-values resulting from this analysis are shown. All interactions show significant differences to the KWS Cashel susceptible control, with all other lines having lower average pycnidia coverage than KWS Cashel.

Table 3: The average symptoms on inoculated leaves of each wheat genotype.

Wheat Genotype	Overall level of resistance	*Stb* genes	No. *Z. tritici* isolates tested	Average No. days to appearance of Symptoms	Average No. of days to appearance of pycnidia	Average final % of inoculated leaf area covered by chlorosis/necrosis	Average final % of inoculated leaf area covered by pycnidia
Taichung 29	Low	*None known*	68	13.0	16.4	97	21
Riband	Low	*Stb15*	90	14.2	17.7	79	23
KWS Cashel	Low	*None known*	85	14.7	17.6	84	36
Synthetic 6X	High	*Stb5*	70	15.4	25.3	50	1
Synthetic M3	High	*Stb16q, Stb17*	44	15.7	No pycnidia developed*	34	0
Kavkaz-K4500	High	*Stb6, Stb7, Stb10, Stb12*	65	17.7	No pycnidia developed*	22	0
Tadinia	Intermediate	*Stb4, Stb6*	71	17.1	19.6	55	9
Estanzuela Federal	Low/Intermediate	*Stb7*	62	14.1	19.1	85	10
Israel 493	High	*Stb3, Stb6*	74	13.6	22.7	59	1
TE9111	High	*Stb6, Stb7, Stb11*	84	17.8	20.7	31	1
Bulgaria 88	Intermediate/High	*Stb1, Stb6*	38	16.8	25.0	50	3
Veranopolis	Intermediate	*Stb2, Stb6*	37	15.9	23.2	47	6
Synthetic M6	Intermediate	*Stb8*	41	16.5	22.7	57	10
Tonic	Low/Intermediate	*Stb9*	29	15.1	21.1	78	15
Salamouni	Intermediate	*Stb6, Stb13, Stb14*	31	17.3	22.8	50	3
Balance	Intermediate	*Stb6, Stb18*	46	16.6	23.7	61	3
Lorikeet	High	*Stb6, Stb19*	31	18.9	No pycnidia developed*	22	0

* No visible pycnidia at time of assessment

Table 4: The proportion of *Z. tritici* isolates that did not generate symptoms of each type on each wheat genotype in any interaction.

Wheat genotype	Overall level of resistance	*Stb* genes	No. *Z. tritici* isolates tested	% *Z. tritici* isolates that did not induce chlorosis/ necrosis	% *Z. tritici* isolates that did not sporulate
Taichung 29	Low	*None known*	68	0	29
Riband	Low	*Stb15*	90	0	14
KWS Cashel	Low	*None known*	85	0	15
Synthetic 6X	High	*Stb5*	70	11	93
Synthetic M3	High	*Stb16q, Stb17*	44	32	100
Kavkaz-K4500	High	*Stb6, Stb7, Stb10, Stb12*	65	26	97
Tadinia	Intermediate	*Stb4, Stb6*	71	3	48
Estanzuela Federal	Low to intermediate	*Stb7*	62	0	34
Israel 493	High	*Stb3, Stb6*	74	5	92
TE9111	High	*Stb6, Stb7, Stb11*	84	10	92
Bulgaria 88	Intermediate to high	*Stb1, Stb6*	38	0	74
Veranopolis	Intermediate	*Stb2, Stb6*	37	3	68
Synthetic M6	Intermediate	*Stb8*	41	5	41
Tonic	Low to intermediate	*Stb9*	29	0	45
Salamouni	Intermediate	*Stb6, Stb13, Stb14*	31	3	68
Balance	Intermediate	*Stb6, Stb18*	46	0	80
Lorikeet	High	*Stb6, Stb19*	31	35	100

to the inoculation procedure (leading to the overestimation of symptoms), this phenotypic trait was considered less reliable then Trait 4.

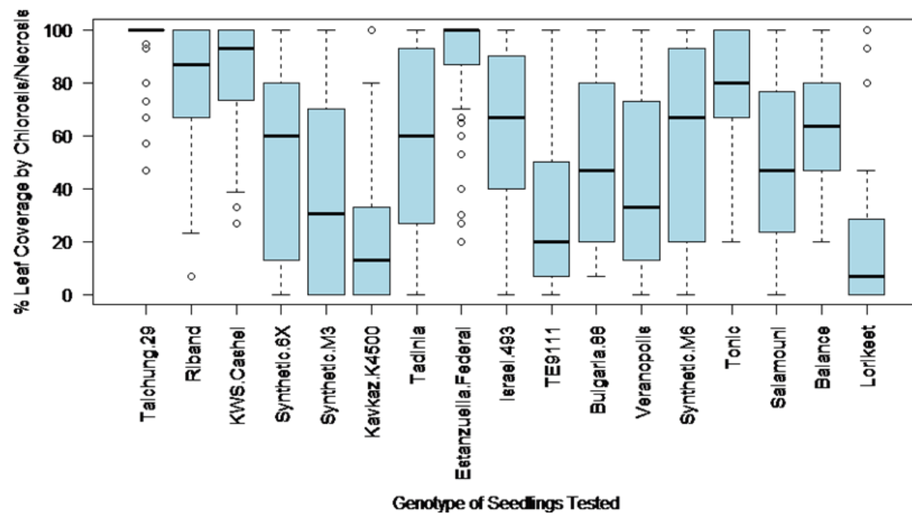

Figure 1: The variation in leaf coverage by chlorosis and necrosis induced by different *Z. tritici* isolates as observed at 28 days post inoculation on each of the 17 studied wheat genotypes.

Trait 4: Inoculated leaf coverage by pycnidia

The final percentage of the inoculated area of the leaf covered by pycnidia at 28 days post inoculation was expected to provide an estimate of the extent to which each isolate of the pathogen could effectively complete its asexual reproductive cycle on each wheat genotype, which is likely to be the strongest measured indicator of the capacity of each isolate to generate an epidemic in the field. The percentage of leaf area covered by pycnidia was more consistent for wheat genotype – *Z. tritici* isolate interactions than Trait 3, and thus became the primary factor used to differentiate between disease resistance and susceptibility. The variation in pycnidia coverage levels for each wheat genotype over the range of *Z. tritici* isolates tested is shown in Figure 2. The highest average level of pycnidia coverage was 36% in KWS Cashel, while the lowest were 0% for Synthetic M3, Kavkaz-K4500 and Lorikeet.

The percentage leaf coverage by pycnidia in all other tested genotypes was significantly lower compared to the susceptible control KWS Cashel in two-tailed paired Student's *t*-tests (Table 2). This includes Taichung 29, which contains no known *Stb* genes. This may be due to possible differences in the plant leaf architecture resulting in fewer fungal penetration events, or potentially due to previously unidentified minor-effect resistance QTL(s). This indicates that all other wheat genotypes tested were significantly more resistant than KWS Cashel

using this phenotypic trait, which is the most directly related to these isolates' ability to cause an epidemic in the field.

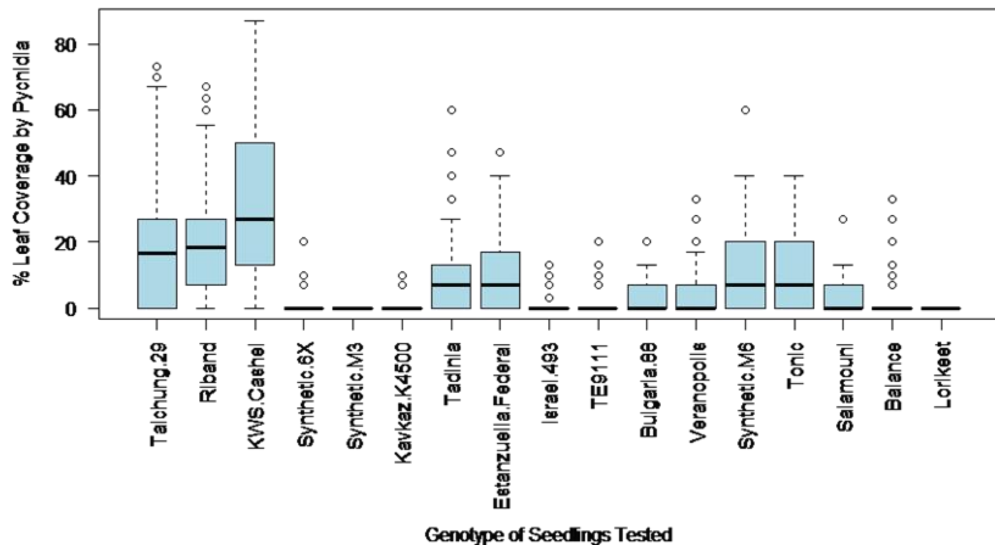

Figure 2: The variation in leaf coverage by pycnidia at 28 days post inoculation with different *Z. tritici* isolates on each of the 17 studied wheat genotypes.

Comparative assessment of average levels of *Z. tritici* resistance in wheat genotypes based on four phenotypic traits

It should be emphasised that these results are calculated by averaging disease assessment scores from many individual *Z. tritici* isolates tested for each wheat genotype. Resistant genotypes, such as TE9111, Kavkaz-K4500 and Synthetic 6X were generally resistant to almost all isolates tested. However, genotypes such as Tadinia had far more variable resistance, with some isolates inducing high infection scores across all assessment criteria while others produced no symptoms, generating intermediate average scores (Table 3). This suggests that these resistances are specific to fungal isolates carrying particular avirulence factors (a "gene-for-gene" relationship) which are each present in only some UK *Z. tritici* isolates. This also indicates that the underlying resistance mechanisms are highly effective when recognition occurs, acting to halt infections before visible symptoms can be caused in many cases even against isolates with the potential to be highly virulent on wheat lines lacking such resistance genes.

In most cases, wheat genotypes displayed similar symptom severity across all measurements. However, for some genotypes (e.g. Israel 493) the development rate and final percentage leaf coverage of chlorosis were high compared to the final percentage of pycnidia leaf coverage. Similarly, early chlorosis followed by high resistance to pycnidia development were seen in Synthetic 6X and Synthetic M3,

although not all *Z. tritici* isolates stimulated visible chlorosis development in these lines (e.g. RResHT-8 and RResHT-10 show 33-86% chlorosis in both Synthetic 6X and Synthetic M3, whereas RResHT-21 and RResHT-24 show 0-7% chlorosis in both lines).

The results obtained in this study demonstrate great variability between the resistances of different wheat lines to UK *Z. tritici* isolates. As expected, wheat lines containing no known *Stb* genes are by far the least resistant group, with almost all tested isolates being highly virulent against KWS Cashel and Taichung 29. This indicates that only very low levels of non-specific resistance for *Z. tritici* are present in most wheat lines.

Overall, in addition to the wheat genotypes Taichung 29 and KWS Cashel (no known *Stb* genes), Riband (*Stb15,* common and widely broken in Europe (Arraiano *et al.*, 2009)) was more susceptible than other lines. Estanzuela Federal (*Stb7*) also showed low resistance to most isolates tested (though higher than in fully susceptible lines for pycnidia coverage), indicating that UK *Z. tritici* populations are virulent towards *Stb7* and *Stb15*. Tonic also showed relatively low resistance, although it was less susceptible than Taichung 29, KWS Cashel or Riband.

Israel 493 (*Stb3* and *Stb6*) and TE9111 (*Stb6, Stb7* and *Stb11*) showed relatively high levels of resistances, indicating that *Stb3* and *Stb11* could be of high potential interest to UK breeders. The synthetic and synthetic-derived lines Synthetic 6X, Synthetic M3 and Lorikeet also demonstrated high levels of resistance, likely due to their novel *Stb* resistance genes (*Stb5*, *Stb16q* and *Stb17*, and *Stb19,* respectively). Kavkaz-K4500 (*Stb6*, *Stb7, Stb10* and *Stb12*) provides good levels of resistance, likely due to the presence of *Stb10* and *Stb12* (as *Stb6* is known to be widely broken and *Stb7* has been shown to be ineffective due to the susceptibility of Estanzuela Federal).

The lines Tadinia, Balance, Synthetic M6, Bulgaria 88, Veranopolis, and Salamouni had more intermediate average levels of resistance, indicating that the genes *Stb1, Stb2, Stb4, Stb8, Stb9, Stb13, Stb14* and *Stb18* all provided partial resistance, or provided resistance to some but not all *Z. tritici* isolates tested. These *Stb* genes could also be interesting to breeders as most would take relatively little effort to move into new wheat cultivars, and are likely to produce reasonable levels of resistance under field conditions (where inoculum levels will be lower than in these screens). However, the genetic variability of *Z. tritici* in the field suggests that individually these genes are unlikely to offer stable resistance, as at least one *Z. tritici* isolate will be virulent against each. It is likely that these genes would have to be stacked to provide durable resistance, slowing and complicating the breeding process.

It was notable that Riband, Estanzuela Federal and Tonic possessed the least resistance among *Stb* gene containing genotypes. Riband showed the highest levels of pycnidia amongst the lines possessing at least one *Stb* gene. This is likely to be because *Stb15* is known to have been widely present in European wheat lines historically (Arraiano *et al.*, 2009), meaning that the local *Z. tritici* populations have adapted to its presence. Tonic had the second highest levels of pycnidiospore production (data not shown) and Estanzuela Federal having the second highest levels of pycnidia coverage. This suggests that the *Stb* genes found in these lines (*Stb7*, *Stb9* and *Stb15*) do not provide good resistance to most *Z. tritici* isolates present in the UK population and should be considered low priority breeding targets for UK wheat lines (although these genes may be more effective against *Z. tritici* populations in other parts of the world).

Identification of preferential breeding targets for maximising the durability of STB resistance genes

The broadest complete resistances were found in Synthetic M3, Kavkaz-K4500, TE9111 and Lorikeet. These genotypes collectively contain *Stb6, Stb7, Stb10, Stb11, Stb12, Stb16q, Stb17,* and *Stb19*. However, the *Z. tritici* isolates used in this test were selected from a dataset of isolates known to be virulent against lines containing *Stb6*. Additionally, *Stb6* and *Stb7* were present in less resistant lines (e.g. Veranopolis and Estanzuela Federal), likely indicating that these *Stb* genes contributed minimally to the resistances of these cultivars.

In Kavkaz-K4500 and Synthetic M3, *Stb10* is paired with *Stb12* and *Stb16q* is paired with *Stb17,* respectively. As none of the genotypes tested contained these genes individually, it is difficult to determine from these results what proportion of the Kavkaz-K4500 and Synthetic M3 resistances each *Stb* resistance gene in these lines was responsible for. It should be noted that previous experiments and field observations demonstrate that *Stb16q* provides extremely broad resistance to the UK *Z. tritici* population present in 2015-2017 (Tabib Ghaffary *et al.*, 2012; Saintenac *et al.*, 2021) whilst *Stb17* was demonstrated to act primarily in adult plants, older than the seedlings used in this study (Tabib Ghaffary *et al.*, 2012). This indicates that *Stb16q* is likely to be responsible for most of the resistance seen in Synthetic M3.

Further experimentation using nearly isogenic lines containing each of these genes individually will aid determining for certain which provide the broadest resistance – until such time as this work is completed, *Stb5, Stb11* and *Stb19* appear to be the highest priority breeding targets found in these bioassays.

Identification of a class of STB resistance responses associated with strong early leaf chlorosis and reduced pycnidia production

An examination of the level of resistance to different symptoms of *Z. tritici* infection in each wheat genotype also reveals a broader category of potentially interesting *Stb* genes that show high levels of resistance to pycnidia development but do not protect from the early development and high final coverages of chlorotic and necrotic symptoms on the leaves. For example, Israel 493 (containing *Stb3* and *Stb6*) shows the sixth highest average symptom coverage score of all tested genotypes (the fourth highest amongst genotypes possessing at least one *Stb* gene), yet has negligibly low average levels of pycnidia coverage, similar to what is shown in Figure 3. This could indicate the presence of resistance genes that act specifically to disrupt the pycnidia formation stage of fungal pathogen development, or the presence of resistance pathways which cause chlorosis as a side effect less damaging then allowing the fungus to grow unimpeded, although it seems unlikely that chlorosis is directly tied to the resistance mechanism as chlorosis is usually linked with cell death and *Z. tritici* is primarily necrotrophic.

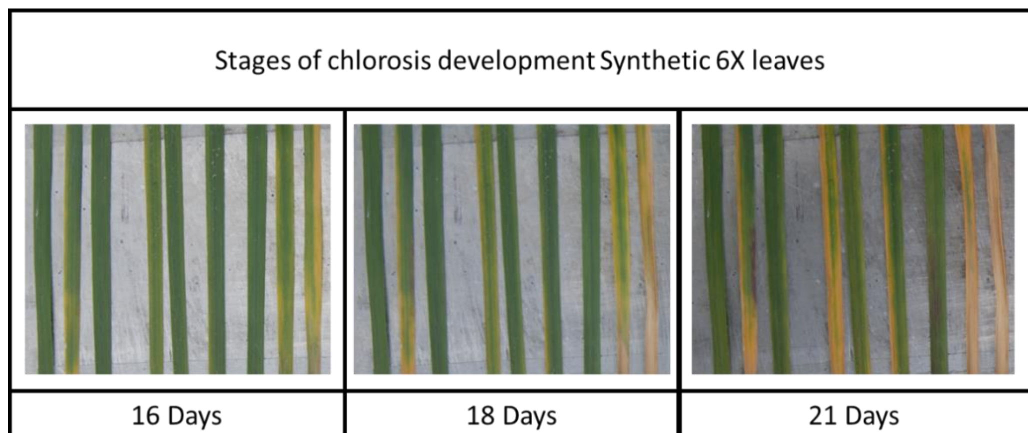

Figure 3: The early chlorosis symptoms and lack of fungal pycnidia observed on Synthetic 6X leaves at three different time-points.

This unusual combination of symptoms could indicate the activation of resistance mechanisms involving a hypersensitive response, likely involving early reactive oxygen species-producing reactions in the chloroplasts (as indicated by the early and strong chlorosis response). This resistance mechanism seems likely to be effective at preventing the spread of a *Z. tritici* epidemic in the field by preventing pycnidia development, although there may also be some loss of photosynthetic potential from individual plants. This could suggest that *Stb3* and other

resistance whose action is associated with high levels of chlorosis genes could provide more durable resistance if deployed in combination with other resistance genes, not associated with chlorosis, as the two different resistance mechanisms would be difficult for any *Z. tritici* isolate to adapt to. However, the utility of these resistances is likely to depend on the level of loss of photosynthetic potential in the field, which cannot easily be estimated from this work, as the high levels of inoculum used to ensure infection here are unrealistic under normal field conditions. Additionally, it is not known which resistance response would be activated against isolates avirulent on wheat genotypes containing both resistance genes associated with chlorosis and those that do not associate with chlorosis. Further experimentation and fieldwork are needed to determine the utility of combining these two mechanistically different types of resistance genes.

Discussion

Zymoseptoria tritici is one of the most important pathogens in the wheat-based agricultural systems of Europe, and chemical defences against it do not seem likely to be durable in the long term. It is therefore vital that breeders be able to effectively utilise *Stb* resistance genes to prevent major epidemics. This study provides data that will help to target UK breeding efforts to the most effective *Stb* resistance genes.

Data provided by field trials can be difficult to standardise due to genetic differences in *Z. tritici* populations locally (Berraies *et al.*, 2013; Mekonnen *et al.*, 2020) and globally. Further issues are caused by the dramatic effect of weather conditions (particularly rainfall) on STB disease development, which can cause large fluctuations in readings between years at the same sites (Ouaja *et al.*, 2020). Additional complexities are added to data analysis by wheat lines with resistance levels that change over the wheat life cycle (e.g. high seedling and low adult resistance) and by imperfect correlations between the levels of different infection symptoms (e.g. necrosis levels and pycnidia counts) (Ouaja *et al.*, 2020). This information is particularly lacking for novel STB disease resistance sources, such as synthetic hexaploid wheats. Overall, the results presented here suggest that the lines Lorikeet (containing *Stb19*) and Synthetic M3 (containing *Stb16q* and *Stb17*) should be of the greatest interest to breeders, as these genotypes were resistant to pycnidia formation from every *Z. tritici* isolate they were challenged with in these bioassays, along with Kavkaz-K4500 (containing *Stb6, Stb7, Stb10* and *Stb12*), Synthetic 6X (containing *Stb5*) and TE9111 (containing *Stb6*, *Stb7* and *Stb11*), which had very high overall resistance. However, Synthetic M3 carries two *Stb* genes, *Stb16q* and *Stb17*. Of these, previous research suggests that *Stb17* is

effective only in adult plants (Tabib Ghaffary *et al.*, 2012), suggesting that the Synthetic M3 resistance is primarily due to the effect of *Stb16q*, which is known to provide broad resistance against *Z. tritici*. However, it should be noted that the resistance provided by *Stb16q* in the field is likely to be less complete than these results suggest, as the bioassays described here used UK *Z. tritici* isolates collected between 2015 and 2017. Since these dates, use of *Stb16q* in elite wheat lines has led to selection for *Z. tritici* isolates capable of virulence against lines containing this resistance, e.g. those found in Ireland and Iran (Dalvand *et al.*, 2018; Kildea *et al.*, 2020), which will likely lead to reductions in the field effectiveness of *Stb16q* over the coming years (as has previously been seen for *Stb6* and *Stb15*). This effect has not yet been noted for the resistance gene *Stb19*, which has not been used in the UK thus far. However, it seems likely that wider use of *Stb19* in elite lines would favour the development of *Z. tritici* isolates capable of breaking this resistance, leading to the loss of efficacy of this resistance gene. It is therefore important that when *Stb19* is used, it is supported by additional genes that provide broad resistance to the local *Z. tritici* population.

The results of this bioassay suggest Kavkaz-K4500 (*Stb6, Stb7, Stb10* and *Stb12*), Synthetic 6X (*Stb5*) and TE9111 (*Stb6, Stb7* and *Stb11*) as good potential sources for these protective *Stb* resistance genes. These genotypes show no pycnidia development from 98%, 96% and 95% of tested *Z. tritici* isolates respectively, with low pycnidia coverages (a maximum of 20% average) from the remaining isolates. All isolates tested against all three genotypes proved avirulent against at least one. As results from Estanzuela Federal and previous research suggest that *Stb6* and *Stb7* provide little or no resistance from UK *Z. tritici* populations (Czembor *et al.*, 2011; Makhdoomi *et al.*, 2015; Stephens *et al.*, 2021), it seems likely that *Stb5, Stb11* and either *Stb10* or *Stb12* are responsible for these resistances. As *Stb10* and *Stb12* were not available for testing in isolation, it was not possible in this study to assess proportion of the total Kavkaz-K4500 resistance was associated with each of these genes. Therefore currently *Stb5* and *Stb11* appear to be the optimal resistances to protect the durability of *Stb19* in future wide use. The long-term effectiveness of the Kavkaz-K4500 resistance despite the widespread use of this genotype in breeding suggests that such pyramids of mutually protective *Stb* genes are likely to be effective in slowing the development of virulence against them in *Z. tritici* populations.

The most useful *Stb* genes identified here are novel genes originating from synthetic hexaploid wheat lines and those that have historically been protected by the presence of multiple resistances in a single breeding line. This may cause issues during the breeding process, as synthetic-derived lines could carry undesirable genes (causing linkage

drag when resistances are transferred to elite lines, possibly reducing yields) and effective resistances may be difficult to identify from wheat lines in which they coexist with several ineffective resistances. The high average resistance of novel lines aligns well with the results of Arraiano and Brown (2006), which found that of 238 wheat genotypes tested, the line with the highest non-specific resistance in their study was the Italian landrace Rieti. Similar results have been found in a number of other studies (Ajaz *et al.*, 2021). Although the resistances identified as broadly effective in this study were highly specific rather than non-specific, both results still indicate that the time *Z. tritici* has been given to adapt to widely used resistances is a vital determining factor in the effectiveness of these resistances. However, the Arraiano and Brown (2006) paper utilised isolates which are now severely outdated and several generations removed from current wild *Z. tritici* populations, along with detached leaf assays, which may cause issues with measuring symptoms such as necrosis coverage (which Arraiano and Brown (2006) did not attempt to monitor). This study used more recent field isolates of *Z. tritici* collected from a more localised region (around the UK) and tested against a smaller set of wheat genotypes, producing a dataset more optimally targeted for identifying resistance genes of interest to breeders in this area. This study also selected wheat genotypes for testing based on the presence of known major resistance genes whereas Arraiano and Brown (2006) aimed to test a broader set of wheat genotypes for any resistance regardless of genetic origin, which together with the more modern *Z. tritici* isolates used in the present study makes it difficult to draw direct conclusions from differences in the average resistances observed.

Resistance to *Z. tritici* is a relatively new target in wheat breeding, meaning that much of the research relating to this pathogen and its interactions with crop plants is still in the early stages and major details of the infection and resistance processes (e.g. potential *Z. tritici* effector impacts on host chloroplast function or the mechanisms of most *Stb* gene for gene resistances) are largely unknown at a molecular level. So far, only *Stb6* and *Stb16q* have been cloned (along with the corresponding fungal effector AvrStb6 recognised by *Stb6*) (Zhong *et al.*, 2017; Saintenac *et al.*, 2018; Saintenac *et al.*, 2021). Much of the research conducted thus far has utilised the model isolate held by most laboratories, IPO 323 – however, this isolate is not reflective of modern field isolates in important ways. For example, IPO 323 is naïve to all modern fungicides and avirulent on cultivars with disease resistance genes that have now been broken down by a large majority of isolates found in the field (e.g. *Stb6*). It is therefore important that novel *Stb* resistance genes be tested more broadly against collections rather than single *Z. tritici* isolates, to assess whether they act sufficiently broadly to be useful in a commercial growing context. The *Z. tritici* isolates utilised in this study were selected from UK fields between the

years 2015 and 2017, and are virulent against *Stb6*. Although these isolates have not been sequenced, the range of different resistance responses they triggered in some wheat genotypes suggests a high level of genetic diversity. This is supported by the well-established genetic diversity of *Z. tritici* even in limited geographic regions (Berraies *et al.*, 2013; Mekonnen *et al.*, 2020; Orellana-Torrejon, Vidal, Gazeau, *et al.*, 2022) and indicates that the results identified here should be broadly applicable to UK *Z. tritici* populations.

Although broadly resistant wheat genotypes often shared resistance to specific *Z. tritici* isolates, no statistically significant associations were found between the virulence of given isolates against any one broad resistance and those isolates' virulence levels against any other broad resistance (data not shown). This suggests that most of the *Stb* resistance genes tested here operate through the recognition of different avirulence factors. No *Z. tritici* isolate tested here was shown to be virulent against all host genotypes assessed in this study. Therefore, it should be possible to develop highly resistant breeding lines by stacking many *Stb* genes. Such gene pyramids would likely improve the durability of all *Stb* genes included (provided that these *Stb* genes were only used in such gene pyramids), as it is much less likely that any given isolate would gain all of the required mutations for virulence at once and thus overcome the resistance. This could be extremely useful in the long term – for example, Kavkaz-K4500 has been considered an STB resistant breeding line for many years and still appeared effective in our experiments, suggesting that combinations of resistance genes that utilise different mechanisms may not only help to increase the durability of each individual gene, but could also be broadly effective due to the collective action of these genes. The use of modern genetic markers and breeding techniques will be necessary to overcome potential obstacles to breeding such as linkage drag and epistasis effects. For example, markers could help track specific resistance genes present in breeding materials derived from genotypes containing multiple such *Stb* resistance genes, and the production of nearly isogenic lines assisted through genotyping using such markers could limit the effect of linkage drag on new breeding lines. However, it is likely that significant breeding work would still be required to introgress the majority of the *Stb* genes examined here into regionally adapted elite breeding lines, as the corresponding disease resistance sources used in this study were originally bred for different environments and growth habits (e.g. Bulgaria 88 is a Bulgarian winter type wheat, whereas Israel 493 is an Israeli spring type wheat) and most are not recent but were developed years or decades ago.

In summary, this study revealed that sufficiently diverse *Stb* genes exist to give broad and durable protection from UK *Z. tritici* isolates to new wheat lines. However, generating this protection in a sustainable

form will require extensive breeding efforts. We identified suitable *Stb* genes to prioritise for pyramiding. However, further work will be necessary to identify modern high-throughput markers such as Kompetitive Allele Specific PCR (KASP) markers (Semagn *et al.*, 2014) for each *Stb* gene of interest to ensure that multiple broadly effective genes can be stacked in a single line (as otherwise epistatic effects may make their presence difficult to confirm), and to produce lines containing each *Stb* gene from highly resistant lines individually for further detailed characterization. There therefore remains much work to be done collaboratively between UK wheat breeders and the scientific community to ensure the desired level of resistance in future wheat.

Chapter 3 - Controlled environment tests on wheat Quasi-NIL lines reveal optimal candidates for *Stb* disease resistance gene pyramids effective against *Zymoseptoria tritici*

Abstract

Septoria tritici blotch disease is one of the most economically important diseases of wheat occurring in Europe and other temperate wheat-growing regions, and is a significant threat to many food systems. Due to a lack of commercial biological controls and increasing fungicide resistance in the causal agent, *Z. tritici*, wheat resistance (*Stb*) genes are currently the most promising method for long-term control of this disease. Unfortunately the lack of genetic information and single-gene phenotypic testing for most *Stb* genes limits breeders' ability to focus breeding programs on optimally effective genes and conduct high-throughput breeding programs. Here, suitably differentiating *Z. tritici* populations are tested against wheat quasi-near isogenic lines (NILs) and segregating populations. Work conducted using segregating wheat populations for resistance genes of interest enabled the development and/or confirmation of new KASP markers for *Stb* genes of high value to breeders (i.e. broadly effective against local *Z. tritici* populations). In particular, KASP markers produced for the *Stb* resistance genes *Stb5*, *Stb10* and *Stb18* were confirmed to be effective and are expected to be useful for tracking high-impact resistances in the field. Tests conducted using quasi-NIL wheat lines enabled the differentiation of the effects of *Stb* genes not commonly tested outside of gene pyramids, demonstrating that a large portion of the durable resistance seen in the wheat line Kavkaz-K4500 appears to be provided by the gene *Stb10*, while confirming relatively little of the resistance seen in the wheat line Synthetic M3 appears to be due to *Stb17* in seedlings. The potential presence of novel resistance QTLs separate from the previously known *Stb* resistance genes in Tadinia and Estanzuela Federal is also suggested from this work.

Contributions

My contributions to this chapter include:

- The growth, maintenance and where necessary purification of all *Z. tritici* isolates used.
- The growth and preparation of all host plants used.
- The inoculation of host plant leaves.
- The phenotyping of inoculated plants throughout symptom development.
- The harvesting of leaves after the 28 day infection period, their high humidity incubation and the performance of the spore counting procedure on these leaves.
- Identification of suitable SNPs and DNA sequences to be covered for some markers using AX- prefixes, from Axiom 35K Breeders Array data.

- Identification of suitable SNPs and DNA sequences to be covered for all markers using chromosome number prefixes, from WAK promotome data.
- The statistical analyses and interpretation of results.
- The writing of this chapter.

Other contributions to this chapter include:

- The initial provision of fungal isolates used by Bart Fraaije.
- The breeding of the original segregating and quasi-NIL host lines used by RAGT Seeds Ltd., initiated before my recruitment to this project due to the time requirements for such breeding.
- Generation of Axiom 35K Breeders Array data by RAGT Seeds Ltd., providing SNP location data on which markers named with the AX- prefix were based, carried out before my recruitment to this project.
- Generation of the WAK promotome sequence dataset by Rothamsted Research, providing SNP location data on which markers named with chromosome number prefixes were based, carried out before my recruitment to this project.
- Development of the markers using the cfn prefix by the Saintenac team (Saintenac *et al.*, 2018; Saintenac *et al.*, 2021).
- Development of the majority of markers using AX- prefixes, from Axiom 35K Breeders Array data.
- Final verification of marker designs using proprietary Kraken and PolyMarker software by RAGT Seeds Ltd.
- Reading of segregating population plant genotypes using KASP markers was conducted by the RAGT Seeds Ltd. high-throughput KASP marker lab.
- Advice from my supervisory team (Kostya Kanyuka, Jason Rudd, Rumiana Ray and Ruth Bryant) throughout the experimental and writing processes.

Introduction

Wheat breeders face many challenges in developing optimal crops that balance maximum yield and reliability for the various wheat growing markets. One of the most difficult challenges is adapting to crop pathogens, which will themselves adapt to overcome the resistances that prevent them from devastating crop yields. In Europe, *Zymoseptoria tritici* (the causal agent of Septoria tritici blotch, STB) is one of the most difficult of these pathogens to mitigate due to the sexual reproductive cycle and typically high genetic diversity of the causal fungus (Berraies *et al.*, 2013; Mekonnen *et al.*, 2020; Orellana-Torrejon, Vidal, Gazeau, *et al.*, 2022). This causes the pathogen to rapidly adapt to novel resistance genes and fungicides (Cools and Fraaije, 2008; van den Berg *et al.*, 2013; Hartmann *et al.*, 2021), creating a need for new and more durable mechanisms to control fungal epidemics.

Unfortunately, effective biological controls for *Z. tritici* have yet to be found, and although chemical controls have traditionally been effective, resistant *Z. tritici* strains have now been identified for every fungicide type used to control this disease (Fraaije *et al.*, 2005; Stammler and Semar, 2011; Estep *et al.*, 2015; Birr *et al.*, 2021; Fouché *et al.*, 2021). Broad, non-specific QTL resistances to STB are also not available at the resistance levels needed to control this disease and maintain reliable crop yields, possibly due to the genetic complexity of this pathosystem historically leading to a dearth of suitable markers for such resistances (Arraiano and Brown, 2006; Miedaner *et al.*, 2020).

Therefore new, durable STB resistance sources are required to protect wheat yields. Unfortunately, previous widely used gene-for-gene resistances (*Stb* resistance genes) have been overcome relatively quickly by *Z. tritici* due to its high genetic diversity and sexual reproductive cycle. Although quantitative resistance genes offering partial resistances against *Z. tritici* isolates may be expected to be more generally durable, these resistances are also far harder to identify and to track through breeding programs in the absence of high quality markers due to their partial effects. As large numbers of these genes would need to be stacked in a wheat line to offer effective STB protection, they have historically been of less interest to both scientists and breeders than the easier to achieve full protection of qualitative resistance genes. Therefore, although this may change in the future as new QTLs are found and genetic markers for their tracking are produced, it is currently important that new gene-for-gene resistances are found which are economically attractive to breeders and can rapidly offer broad protection to wheat crops. It is unlikely that quantitative resistances could provide a replacement sufficiently quickly to protect crop yields in the near future, as many widely used resistances are already broken or showing signs of rapid loss of effectiveness in the field. For example, *Stb6* and *Stb15* have been widely used to protect elite lines in Europe but are now almost entirely ineffective (Arraiano *et al.*, 2009; Stephens *et al.*, 2021). *Stb16q* was introduced relatively recently into French commercial varieties, but already *Z. tritici* isolates have been isolated in France (Orellana-Torrejon, Vidal, Boixel, *et al.*, 2022; Ruth Bryant, personal communication), Ireland (Kildea *et al.*, 2020) and Iran (Dalvand *et al.*, 2018) which are virulent against plants containing this resistance. New highly effective resistances are urgently needed to replace those the pathogen has, or in the near future will, overcome. Additionally, new methods must be found to protect these resistances.

Fortunately the resistances of some wheat breeding lines, such as Kavkaz-K4500, have proven more durable. Kavkaz-K4500 has been used as a resistance source to *Z. tritici* in wheat breeding programs for decades. Generally, these high-durability lines are those that contain

multiple *Stb* genes (e.g. *Stb6, Stb7, Stb10* and *Stb12* for Kavkaz-K4500), although it is difficult to ensure that all resistance genes in such pyramids are inherited by elite descendent lines in a breeding program as these genes are located on different chromosomes and tend to segregate. The durability of Kavkaz-K4500 suggests that the formation of pyramids of resistance genes is likely to be effective at increasing the durability of each resistance gene in the pyramid, likely because a large number of simultaneous independent mutations would be necessary to produce a *Z. tritici* strain capable of virulence against these lines.

Due to this, there are several wheat lines with broad resistance in which it has been difficult to identify which *Stb* genes provide resistance to different *Z. tritici* isolates and which do not. Additionally, potential interactions between multiple *Stb* genes during infection are not well understood, and are difficult to speculate on due to the lack of mechanistic information we have on most *Stb* genes. This can make it challenging for breeders to plan optimised breeding programs for STB resistance, slowing the process and encouraging the use of single large effect resistance genes being introduced to breeding programs from wheat lines that show adequate resistance, potentially even without confirming which resistance gene/s each such line contains. This is likely to contribute to the difficulty of future STB resistance breeding and the lack of durability noted in *Stb* resistance genes.

To make better use of available resources and conserve their effectiveness into the future, it would be highly desirable to produce elite wheat lines possessing multiple broadly effective *Stb* resistance genes. Unfortunately, this creates difficulties for breeders. Many broad *Stb* resistance genes will be epistatic to one another under field conditions, making it very difficult to follow multiple resistances through a population, and thus also making it very difficult to ensure that all resistance genes in a gene pyramid have been inherited at each stage of a breeding program unless markers can be used to confirm the presence of each individual *Stb* resistance gene at each generation. Using artificial *Z. tritici* inoculations instead of field infections will not solve this issue easily, as each *Z. tritici* isolate used would need to be avirulent against only one of the *Stb* genes undergoing testing. This is because the presence of one resistance gene to which a *Z. tritici* isolate was avirulent in a wheat line would make it impossible to use that isolate to test for the presence of further *Stb* genes in that wheat line, as gene-for-gene resistances are generally sufficiently complete that there would be no noticeable increase in resistance against an already avirulent fungal isolate obtained from adding additional resistances.

The fact that most of the known *Stb* resistance genes are located on different chromosomes to one another also makes it impossible to

solve this issue by producing a stable linkage group of resistances in which individual *Stb* resistance genes will not segregate independently between descendent lines when crossing cultivars to produce new elite lines in a breeding program. Genetic modification techniques could be used to solve this issue, but this is not practical in Europe due to restrictive regulations effectively banning this technology from practical use in the food production industry (Callaway, 2018).

These issues are exacerbated by the fact that many of the most broadly effective *Stb* resistance genes are derived from synthetic lines. It is presumed that the novelty of these genes explains why *Z. tritici* populations do not have widespread virulence against them – for example, when *Stb16q* was initially discovered in the synthetic line Synthetic M3, it was a broadly effective resistance in the field across Europe (Tabib Ghaffary *et al.*, 2012), but as lines containing this resistance have been used in France, Iran and Ireland, virulent isolates have become more common in the *Z. tritici* population (Dalvand *et al.*, 2018; Kildea *et al.*, 2020). This implies that the more widespread the use of a resistance gene becomes, the higher the likelihood of virulent isolates emerging against it. Unfortunately, many reproductive generations are likely to be necessary in breeding programs to separate high-value novel resistances against which little virulence is currently likely to exist from the linkage drag associated with synthetic lines and landraces, and many *Z. tritici* strains are already likely to have adapted to any resistance present in modern elite lines.

Therefore breeders will need new tools to manage such breeding programs efficiently. High throughput modern markers will be necessary to follow multiple *Stb* resistance genes through the large scale breeding programs and high number of wheat generations needed to stack multiple novel resistance genes in a single wheat line, while simultaneously the linkage drag associated with each resistance gene due to their likely origins in landraces and synthetic wheat lines. To this end, a variety of Kompetitive Allele Specific PCR (KASP) markers (Semagn *et al.*, 2014) were designed and/or verified for multiple *Stb* resistance genes known to be broadly or partially effective against UK *Z. tritici* isolates (Tidd *et al.*, 2023). Such markers could be of use to UK and European breeders.

The efficacy of several promising *Stb* resistance genes identified as potentially broadly effective against UK fungal populations in earlier work (Tidd *et al.*, 2023) was also tested to aid in the selection of optimal combinations of *Stb* resistance genes for inclusion in a durable resistance gene pyramid against STB. This data is expected to be particularly useful for breeders interested in wheat lines containing multiple *Stb* genes, for whom it will be important to determine which of the *Stb* resistance genes present in such lines can independently provide sufficient resistance to be worth pursuing in breeding

programs. This may be challenging, as more broadly effective *Stb* resistance genes may be epistatic to less broadly effective *Stb* resistance genes in the field, and breeders will need to understand which of the *Stb* resistance genes present provide the majority of the STB resistance observed in lines such as Kavkaz-K4500 (Tidd *et al.*, 2023). The testing process described here utilised wheat NIL lines produced through the backcrossing of partially or broadly STB resistant wheat lines to the susceptible wheat line KWS Cashel, which possesses no known *Stb* resistance genes. These NIL lines may additionally be directly useful in future breeding programs, as much of the linkage drag associated with the synthetic resistance lines and landraces used as resistant parents (e.g. Synthetic 6X, containing *Stb5*) is likely to have been removed through this backcrossing to KWS Cashel, reducing the level of pre-breeding required for these lines to be introduced into elite breeding programs.

The primary hypotheses of this work were: (1) That the major *Stb* resistance genes identified in Tidd *et al.* (2023) would be inherited in accordance with the assumptions of Mendelian genetics; (2) That it would be possible to produce KASP markers capable of following each of these resistance genes of interest through a segregating population and thus accurately predicting the STB resistance of F_3 descendent wheat lines; and (3), that in broadly resistant wheat lines containing multiple known *Stb* resistance genes, there would be variation in the levels of resistance provided by each such resistance gene individually;

Materials and Methods

Library of fungal isolates

Z. tritici isolates obtained from Bart Fraaije (Rothamsted Research) were grown on 7% (w/v) YPD agar (Formedium) plates containing 1 unit of penicillin and 1 µg/mL streptomycin (Sigma-Aldrich) to remove bacterial contamination. Approximately 25 µl of original *Z. tritici* glycerol stocks were used per plate. Inoculated plates were incubated at 16ºC for four to seven days before the fungus was harvested using a sterile loop into 50% (w/v) glycerol and stored at -80ºC. This was then repeated using antibiotic free (otherwise identical) plates to ensure the fungi were not stressed. Fungi from antibiotics-free plates were harvested and stored identically. Large amounts of fungal material was harvested in this initial harvesting in order to ensure that additional bulking up of fungal material was not generally necessary. This is expected to have prevented genetic drift in the fungal isolates used during the experiments by preventing the passage of sufficient generations to cause this issue. Once pure stocks had been produced, extracts from the same stock (stored consistently at -80°C to ensure

that it did not deteriorate) were plated as needed to grow new inoculum for each isolate.

Where bacterial contaminants proved resistant to the antibiotics used, contaminated glycerol stock was diluted (approximately by a factor of 100, depending on concentration), allowing individual colonies to form from single cells. Suitable uncontaminated colonies were harvested into 50% glycerol and re-plated to produce pure stocks. This required stocks to be re-plated twice, once during the dilution step to allow selection of a pure colony and once more to allow harvesting of the purified fungal material. Large amounts of material where then harvested and stored as before to minimise the need for re-plating and the opportunity for genetic drift.

Production of KASP Markers

Initial KASP marker sets were produced using data from the Axiom 35K "breeders" array and the locations of previously known genetic markers for genes of interest (provided by RAGT Seeds Ltd.). The SNP IDs and chromosome positions of older marker types known to follow *Stb* genes of interest in previous breeding programs were arranged with the results of the Axiom 35K array data, and suitable KASP marker sites from near the previous marker locations were selected as suitable test locations for new markers. Where possible, new marker sites were chosen to show sequence variation between the KWS Cashel susceptible parent and the relevant resistance donor parent only (i.e. with other wheat containing alternate *Stb* resistance genes lines possessing the same sequence as the susceptible KWS Cashel), to ensure that the markers produced could be used to follow resistance genes precisely even in breeding programs involving multiple resistance sources. Markers designed based on this data have names prefixed with AX.

Unfortunately, many of the markers produced from the 35K array were shown not to work as intended during the RAGT Seeds Ltd. breeding process. Therefore additional markers were produced using data produced by the Wheat Genetics Improvement Network (WGIN) program, pre-existing at Rothamsted Research (Kostya Kanyuka, personal communication). This data was produced through a capture of the wheat WAK gene promotome, which was selected based on the discovery that the resistance gene *Stb6* is a WAK gene (Saintenac *et al.*, 2018). This data was produced using sequence similarity to known WAK genes to identify similar sequences in multiple genomes from several different sequenced wheat cultivars. Promoters linked to these genes were identified using conserved features, amplified and sequenced. These sequences were aligned using the Chinese Spring variety as a backbone (the genome assembly used was the IWGSC

Refseq v1.0 assembly (International Wheat Genome Sequencing Consortium, 2018)). Markers designed from this data are prefixed with EC, or do not have a specific prefix.

To identify suitable SNPs from this data, the genetic locations of known markers for *Stb* genes of interest were identified (as with the Axiom data) and located in the genomic data of wheat lines of interest. Sequences of the KWS Cashel susceptible parent and the resistance donor parents were aligned to Chinese Spring in the Integrative Genomics Viewer program (Robinson *et al.*, 2011). Where possible, the sequences of other lines of interest were included in the alignment to enable the selection of SNPs unique to sources of the relevant Stb resistance gene.

The sequence alignment was then visually scanned in both directions from the known marker site for SNPs between the KWS Cashel genome and the genomes of each line of interest, out to distances of over 30,000,000 bp when necessary. Preference was given to SNPs unique to resistance sources that were covered by a high number of reads, although where choice was limited any available SNP was used. SNPs between KWS Cashel and lines of interest at a reasonable distance from each other were identified and the surrounding genetic sequence was confirmed using the *T. aestivum* genome in Ensemble plants at the desired location (Yates *et al.*, 2022). This genome is constructed from the IWGSC Refseq v1.0 assembly (International Wheat Genome Sequencing Consortium, 2018). These sequences and the known SNPs were then used to design KASP markers using the programs Kraken^{TM} (LGC genomics, UK) and PolyMarker (Ramirez-Gonzalez *et al.*, 2015) by the RAGT marker laboratory. Resultant markers were purchased and labelled with a k or p suffix, respectively. See Supplementary Table 1 for a full list of the markers described, the *Stb* resistance genes for which each marker acts as a reporter, the DNA sequences of these markers, and the data from which each marker was produced.

A small number of additional KASP markers were taken from previous papers on the cloning of *Stb6* (Saintenac *et al.*, 2018) and *Stb16q* (Saintenac *et al.*, 2021). The markers cfn80023, cfn80025 and cfn80030 were taken from Saintenac *et al.* (2018), and the markers cfn80031 and cfn80044 were taken from Saintenac *et al.* (2021). Table 1 contains a list of the *Stb* resistance genes tracked in this study, with details of the markers development targeted to these resistances and the wheat lines in which they were tracked.

Table 1: Showing the *Stb* resistance genes tracked through segregating populations in this study, the wheat lines in which they were tracked, and the data used to produce the KASP markers used to track each.

Stb resistance gene tracked	Resistant parent of wheat segregating population used	Number of successful KASP markers used	Data from which KASP markers were developed
Stb5	Synthetic 6X	9	Rothamsted Research WAK Promotome Data and RAGT Seeds Ltd. 35K Axiom Breeders Array data
Stb6	Kavkaz-K4500 and Bulgaria 88	3	Saintenac *et al.* (2018)
Stb7	Estanzuela Federal and Kavkaz-K4500	5	RAGT Seeds Ltd. 35K Axiom Breeders Array data
Stb10	Kavkaz-K4500	2	RAGT Seeds Ltd. 35K Axiom Breeders Array data
Stb11	TE9111	1	RAGT Seeds Ltd. 35K Axiom Breeders Array data
Stb12	Kavkaz-K4500	3	RAGT Seeds Ltd. 35K Axiom Breeders Array data
Stb13	Salamouni	3	RAGT Seeds Ltd. 35K Axiom Breeders Array data
Stb14	Salamouni	4	RAGT Seeds Ltd. 35K Axiom Breeders Array data
Stb16q	Synthetic M3	2	Saintenac *et al.* (2021)
Stb17	Synthetic M3	2	RAGT Seeds Ltd. 35K Axiom Breeders Array data
Stb18	Balance	16	Rothamsted Research WAK Promotome Data

Inoculation of wheat plants

Z. tritici isolates used in inoculations were plated out on antibiotic-free YPD agar plates and grown for four to seven days at 16ºC. Fungal blastospores were then harvested using sterile loops into 5mL of 0.1% Silwet L-77 surfactant in H_2O and diluted to a concentration of 10^7 spores per mL using the average of two replicated measurements from a haemocytometer (Bright-line). High concentrations and the presence of a surfactant are not reflective of field conditions, but were included to encourage rapid infection to reduce the time needed per bioassay.

Plants were grown for approximately three weeks (adapted for variable growth rates where necessary) at 16-hour day, 8-hour night cycles under halogen or white LED lamps (pink LEDs were trialled and found to prevent proper infection development) at a temperature of 21°C and ambient humidity. After inoculation, these plants were transferred to 17°C and the same day/night cycle. The second leaf was inoculated where possible, but for some cultivars (e.g. Israel 493) the third leaves were used due to their larger size.

Cotton buds (COPAN, plain swab, sterile wooden applicator, cotton tip) were used to inoculate each spore suspension onto leaves of three plants of each wheat line (approximately four strokes per leaf, ensured an even layer of moisture on leaf surface). Non-inoculated leaves were trimmed to ensure light access to inoculated leaves.

After inoculation, plants were placed in high humidity boxes for three days to encourage pathogen development before being removed. This inoculation process was copied from that used in Tidd *et al.* (2023).

Visual symptom assessments

Necrosis, chlorosis and pycnidia development symptoms were assessed visually. Assessment of the rate of symptom and pycnidia development began ten days after seedling inoculation by *Z. tritici* for each plant. Assessments were then carried out three times a week at regular intervals until 28 days after the initial inoculation date. Leaf status was recorded as no infection (i.e. clean), chlorosis present (showing yellow chlorotic tissue but which had not yet progressed to necrosis), necrosis present (where necrotic lesions were visible), chlorosis with pycnidia (chlorotic symptoms present with small black pycnidia visible on the inoculated leaf surface) or necrosis with pycnidia. The first date on which chlorosis or necrosis was seen was used to determine the “days until symptom development” symptom value, while the date on which pycnidia were first noted was used to determine the “days until pycnidia development” symptom value. Photographs were taken at each check in case needed for later verification of results.

At 28 days post infection, before leaves were harvested for the pycnidia spore count measurements, the “percentage leaf area covered by symptoms” and “percentage leaf area covered by pycnidia” were visually assessed. The values for each leaf were rounded to 0, 20, 40, 60, 80 or 100% for each leaf. Photographs were taken in case needed for later verification of results.

Pycnidia spore counts

After 28 days post infection, inoculated leaf regions were harvested into 15 mL Falcon tubes (one for the three leaves of each line/isolate interaction). A 2 cm X 2 cm X 1.5 cm plug of absorbent cotton wool pre-wetted with 3 mL of deionised H_2O was used to provide a humid environment in each tube (encouraging pycnidia to push through stomata and ooze pycnidiospores) for 2 days before measurement.

Six mL of 0.01% Tween 20 surfactant (Croda International Plc, Snaith, UK) in H_2O was then added to each tube, and tubes were vortexed for 75 s to wash spores from leaf surfaces into the liquid. The optical density at 600 nm (OD_{600}) of one mL of the resulting suspension was measured using a spectrophotometer CARY 50 (Varian, London, UK).

The spore suspensions giving the highest OD_{600} were used to produce standard curves to convert OD_{600} ratings to spores/mL. This required a series of standard dilutions (2x, 4x, 8x, 16x and 32x) of the spore suspension in 0.01% Tween 20 in H_2O to be measured with the spectrophotometer and haemocytometer (two haemocytometer measurements were averaged to provide the measurement used). In most cases curves were approximately linear, so the formula of the linear trendline (generated in Microsoft Excel) was used in conversions. Supplementary Figure 2 demonstrates the relationships between OD_{600} and haemocytometer spore count readings for a large set of leaves.

Genotyping

In genotyping tests, F_3 seeds derived from crosses between KWS Cashel and the wheat line of interest underwent pre-germination on sand (sterilised at 200ºC for several hours and cooled before being poured in a half centimetre thick layer in a seed tray). Seeds were placed on top of the sand and lightly watered. The tray was then placed in a closed black plastic bag to maintain high humidity during germination for three days.

Once germination began, germinating seeds were transferred into individual cells (one germinated seed per cell) of 20 to 60 cell seed trays filled with soil (Rothamsted prescription mix, containing 75% medium grade (L&P) peat, 12% screened sterilised loam, 3% medium grade vermiculite and 10% grit (5mm, screened, lime free) with various

standard micronutrients). Plants were grown until the second leaf was fully expanded, at which point the first leaf was harvested, freeze-dried and sent to RAGT Seeds Ltd. for KASP genotyping, using the specialist facilities of the high throughput KASP marker lab available there. The results of this genotyping were provided for analysis in simple Excel table format. Each genotyped plant was provided with a unique identifier for each test set, and for each marker tested on each such plant the fluorescence response from the KASP marker was provided as one of three codes (e.g. A:A for the expected resistant parental marker type, a:a for the expected susceptible parental marker type and A:a for a heterozygote in which both fluorophores responded).

The second leaves of test plants were inoculated with fungal spores using a cotton-wool bud. Additional leaves were trimmed regularly, and inoculated plants were monitored for disease development. After 28 DPI, disease symptoms were assessed and the results compared to genotypic readings, to evaluate the predictive power of each marker. Attempts were made to calculate genetic distances between markers and candidate *Stb* genes were calculated based on the recombination frequencies, but were generally not considered reliable due to errors in the relative estimates of known marker positions and orders (data not shown), assumed to be caused by insufficient sample sizes, marker numbers and/or levels of association for the accurate calculation of such genetic distances.

Statistical analysis

Statistical analysis of KASP marker correlation to observed symptoms was carried out using MapDisto software (Lorieux, 2012). To do this, phenotyping and genotyping data was recorded and matched manually in an excel spreadsheet before being transferred to MapDisto. Groups were found in this data using the inbuilt MapDisto tools. Groups were then ordered, and the order rippled to identify the most likely data arrangement, also using the MapDisto tools provided. The p-values for correlation between genotype reported by each KASP marker and symptom levels of inoculated leaves from the corresponding plants were then calculated using ANOVA tests (using the MapDisto QTL/ANOVA1 option).

For the assessment of symptom phenotype-genotype relations in NIL lines, R (R Core Team, 2017) and the Microsoft Excel data analysis tool were used. The results generated using these tools (see results section) were considered sufficiently clear that additional statistical analysis have not been included.

In total, 516 segregating F_3 plants and 472 BC_3 NIL plants were successfully tested in these experiments. More detailed breakdowns of the seedling numbers used in each experiment are given in the Results

and Discussion section. These sets were considered sufficient to provide statistically meaningful results, as illustrated by the statistically significant correlations identified between at least one KASP marker and disease symptom phenotype for all segregating populations, except TE9111 and Bulgaria 88 (due primarily to the limited numbers of effective markers available in both cases).

Results

Estanzuela Federal x KWS Cashel F_3 mapping population analysis

The F_3 segregating population produced from crossing the resistance donor Estanzuela Federal with KWS Cashel was used to test KASP markers flanking *Stb7*. We tested seven markers (two of which produced no usable response) against 20 control plants of each parental line and 107 F_3 hybrids. Of the five phenotypes measured, only the days taken to pycnidia development was statistically significantly correlated to the reading from any KASP marker (P=0.045 with the marker AX-94475129_k and P=0.043 with the marker AX-94780124_k), as shown in Table 2. This is likely due to the lack of clear phenotypic differentiation between the parental lines following inoculation with the chosen isolate, with only two phenotypes (final pycnidia coverage and spore count per leaf, P=0.0025 and P=2X10^{-5} respectively) giving significantly different results in a single factor ANOVA between the parental lines.

Synthetic 6X x KWS Cashel F_3 mapping population analysis

The F_3 segregating population produced from crossing the resistance donor Synthetic 6X with KWS Cashel was used to test KASP markers designed to follow *Stb5*. Thirteen markers were tested successfully (using 72 F_3 individuals, and 24 individuals of each parental line), identifying nine that were significantly associated with final pycnidia coverage and leaf spore count, as shown in Table 3. Only two markers were not significantly associated with any symptom. This gave us a selection of markers highly correlated to Septoria resistance sources near the *Stb5* locus.

Additional testing was conducted using four additional *Z. tritici* isolates (RResHT-24, RResHT-30, RResHT-36 and RResHT-69). Small numbers of plants were used for each of these tests, precluding the identification of significant correlations between markers and resistances (with the exception of chlorosis development when exposed to RResHT-36, for which even with the small sample sizes used, four of the five tested markers showed significant correlations to the development rate and final coverage of chlorosis). The phenotypic measurements from these tests (shown in Figure 1) suggest a general

pattern of F_3 hybrid seedlings possessing resistance averages similar to the resistant Synthetic 6X parent, particularly for the number of spores measured per leaf from spore wash solutions. This could suggest that in addition to *Stb5*, the Synthetic 6X line contains many other resistance QTLs, resulting in significant *Z. tritici* resistance being retained in most or all plants even after multiple breeding generations.

Balance x KWS Cashel F_3 mapping population analysis

The F_3 segregating population produced from crossing the resistance source Balance with KWS Cashel was used to test KASP markers associated with *Stb18* (using 69 F_3 and 51 parental line individuals). All 16 of the markers tested were significantly correlated with symptom levels for at least three of the five symptoms measured, as shown in Table 4. Additional testing was conducted using four additional *Z. tritici* isolates (RResHT-24, RResHT-30, RResHT-36 and RResHT-69). Small numbers of plants were used for each of these tests, precluding the identification of significant correlations between markers and resistances. However, the phenotypic measurements from these tests show that the general pattern of F_3 hybrid seedlings possessing resistance between that of the parental controls remained consistent across multiple *Z. tritici* isolates, suggesting that segregation of resistance genes was occurring as expected in this population. The phenotype results of these experiments are shown in Figure 2.

TE9111 x KWS Cashel F_3 mapping population analysis

The F_3 segregating population produced from crossing the resistance source TE9111 with KWS Cashel was used to test KASP markers associated with *Stb11* (using 60 F_3 individuals and 40 parental line individuals), as *Stb7* had already been shown to be ineffective (through the Estanzuela Federal segregating population tests and previous work (Tidd *et al.*, 2023)). Only one of the five tested KASP markers functioned, and this marker was not significantly associated with any disease symptom phenotype, as shown in Table 5.

Additional testing was conducted using four additional *Z. tritici* isolates (RResHT-24, RResHT-30, RResHT-36 and RResHT-69). Small numbers of plants were used for each of these tests, and none of the tested markers correlated significantly to resistance in any measured phenotype. Figure 3 shows the results of these tests.

Table 2: Table showing the probability of a correlation between each KASP marker and phenotype tested in the Estanzuela Federal x KWS Cashel F_3 mapping population. Green cells show a significant correlation ($P \leq 0.05$, ANOVA test).

Estanzuela Federal			*P*-Values				
Name of KASP Marker Tested	**Position of KASP Marker**	Expected Linked *Stb* Gene	Days to Symptom Development	Days to Pycnidia Development	Final % Symptoms Coverage	Final % Pycnidia Coverage	Spore Count
AX-94918531_k	Position 719178878, Chromosome 4A	*Stb7*	**0.135**	**0.825**	**0.660**	**0.275**	**0.863**
AX-94475129_k	Position 731006478, Chromosome 4A	*Stb7*	**0.120**	**0.935**	**0.998**	**0.045**	**0.658**
AX-94980296_k	Position 743868205, Chromosome 4A	*Stb7*	**0.605**	**0.334**	**0.946**	**0.191**	**0.874**
AX-95175098	Position 740626510, Chromosome 4A	*Stb7*	**0.354**	**0.941**	**0.826**	**0.094**	**0.645**
AX-94780124_k	Position 733433961, Chromosome 4A	*Stb7*	**0.534**	**0.672**	**0.521**	**0.043**	**0.444**

Table 3: The probability of a correlation between each KASP marker tested and phenotype measured in the Synthetic 6X x KWS Cashel F_3 mapping population. Green cells show a significant correlation ($P \leq 0.05$, ANOVA test).

Synthetic 6X			*P*-Values				
Name of KASP Marker Tested	**Position of KASP Marker**	Expected Linked *Stb* Gene	Days to Symptom Development	Days to Pycnidia Development	Final % Symptoms Coverage	Final % Pycnidia Coverage	Spore Count
7D_120312071_Stb5_K	Position 120312071, Chromosome 7D	*Stb5*	**0.007**	**0.056**	**0.763**	**0.059**	**0.327**
7D_120312071_Stb5_K2	Position 120312071, Chromosome 7D	*Stb5*	**0.007**	**0.056**	**0.763**	**0.059**	**0.327**
EC_Stb5_7D_96724451_k	Position 96724451, Chromosome 7D	*Stb5*	**0.057**	**0.225**	**0.682**	**0.034**	**0.007**
EC_Stb5_7D_97759874_k	Position 97759874, Chromosome 7D	*Stb5*	**0.003**	**0.052**	**0.142**	**0.004**	**0.006**
EC_Stb5_7D_96724451_p	Position 96724451, Chromosome 7D	*Stb5*	**0.007**	**0.060**	**0.044**	**0.002**	**0.004**
7D_101717316_Stb5_K	Position 101717316, Chromosome 7D	*Stb5*	**0.006**	**0.050**	**0.231**	**0.010**	**0.004**
7D_101717316_Stb5_K3	Position 101717316, Chromosome 7D	*Stb5*	**0.006**	**0.050**	**0.231**	**0.010**	**0.004**
EC_Stb5_7D_97759874_p	Position 97759874, Chromosome 7D	*Stb5*	**0.003**	**0.052**	**0.142**	**0.004**	**0.006**
AX-94418300_k	Position 88191346, Chromosome 7D	*Stb5*	**0.001**	**0.071**	**0.027**	**0.006**	**0.000**
7D_89597474_Stb5_K	Position 89597474, Chromosome 7D	*Stb5*	**0.001**	**0.056**	**0.025**	**0.006**	**0.001**
7D_89597474_Stb5_K4	Position 89597474, Chromosome 7D	*Stb5*	**0.001**	**0.056**	**0.021**	**0.003**	**0.000**
7D_20495947_Stb5_K	Position 20495947, Chromosome 7D	*Stb5*	**0.227**	**0.096**	**0.612**	**0.062**	**0.120**
7D_20495947_Stb5_K5	Position 20495947, Chromosome 7D	*Stb5*	**0.285**	**0.082**	**0.686**	**0.109**	**0.096**

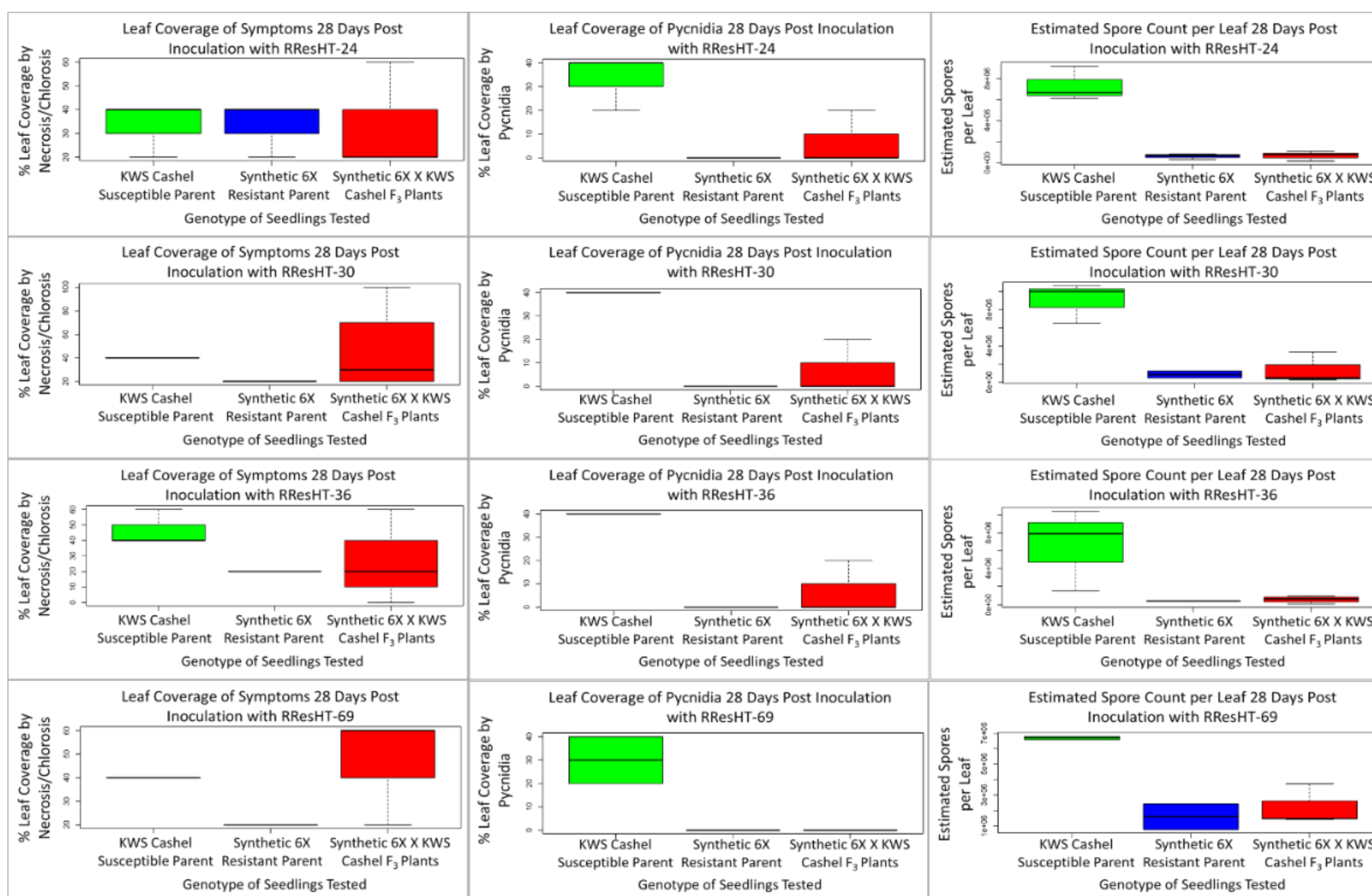

Figure 1: Phenotypes from Synthetic 6X X KWS Cashel F_3 hybrids when tested against four *Z. tritici* isolates. Green boxes represent the susceptible parental line, blue boxes represent the resistant parental line and red boxes represent F_3 hybrid plants.

Table 4: The probability of a correlation between each KASP marker tested and phenotype measured in the Balance x KWS Cashel F_3 mapping population. Green cells show a significant correlation ($P \leq 0.05$, ANOVA test).

Balance			*P*-Values				
Name of KASP Marker Tested	**Position of KASP Marker**	Expected Linked *Stb* Gene	Days to Symptom Development	Days to Pycnidia Development	Final % Symptoms Coverage	Final % Pycnidia Coverage	Spore Count
EC_Stb18_6D_21076387_p	Position 21076387, Chromosome 6D	*Stb18*	**0.082**	**0.077**	**0.000**	**0.000**	**0.000**
EC_Stb18_6D_21076387_k	Position 21076387, Chromosome 6D	*Stb18*	**0.055**	**0.077**	**0.000**	**0.000**	**0.000**
EC_Stb18_6D_25537253_k	Position 25537253, Chromosome 6D	*Stb18*	**0.032**	**0.003**	**0.000**	**0.000**	**0.000**
EC_Stb18_6D_25537253_p	Position 25537253, Chromosome 6D	*Stb18*	**0.045**	**0.003**	**0.000**	**0.000**	**0.000**
EC_Stb18_6D_24377769_p	Position 24377769, Chromosome 6D	*Stb18*	**0.061**	**0.000**	**0.000**	**0.000**	**0.000**
EC_Stb18_6D_24387369_k	Position 24387369, Chromosome 6D	*Stb18*	**0.099**	**0.000**	**0.000**	**0.000**	**0.000**
EC_Stb18_6D_24377838_k	Position 24377838, Chromosome 6D	*Stb18*	**0.058**	**0.000**	**0.000**	**0.000**	**0.000**
EC_Stb18_6D_24387417_k	Position 24387417, Chromosome 6D	*Stb18*	**0.133**	**0.003**	**0.000**	**0.000**	**0.000**
EC_Stb18_6D_24377769_k	Position 24377769, Chromosome 6D	*Stb18*	**0.103**	**0.003**	**0.000**	**0.000**	**0.000**
EC_Stb18_6D_24379384_p	Position 24379384, Chromosome 6D	*Stb18*	**0.361**	**0.182**	**0.000**	**0.000**	**0.000**
EC_Stb18_6D_24378137_k	Position 24378137, Chromosome 6D	*Stb18*	**0.103**	**0.003**	**0.000**	**0.000**	**0.000**
EC_Stb18_6D_24378137_p	Position 24378137, Chromosome 6D	*Stb18*	**0.093**	**0.002**	**0.000**	**0.000**	**0.000**
EC_Stb18_6D_24387417_p	Position 24387417, Chromosome 6D	*Stb18*	**0.103**	**0.003**	**0.000**	**0.000**	**0.000**
EC_Stb18_6D_25727651_k	Position 25727651, Chromosome 6D	*Stb18*	**0.066**	**0.035**	**0.000**	**0.000**	**0.000**
EC_Stb18_6D_26241560_k	Position 26241560, Chromosome 6D	*Stb18*	**0.081**	**0.035**	**0.000**	**0.000**	**0.000**
EC_Stb18_6D_26241560_p	Position 26241560, Chromosome 6D	*Stb18*	**0.023**	**0.035**	**0.000**	**0.000**	**0.000**

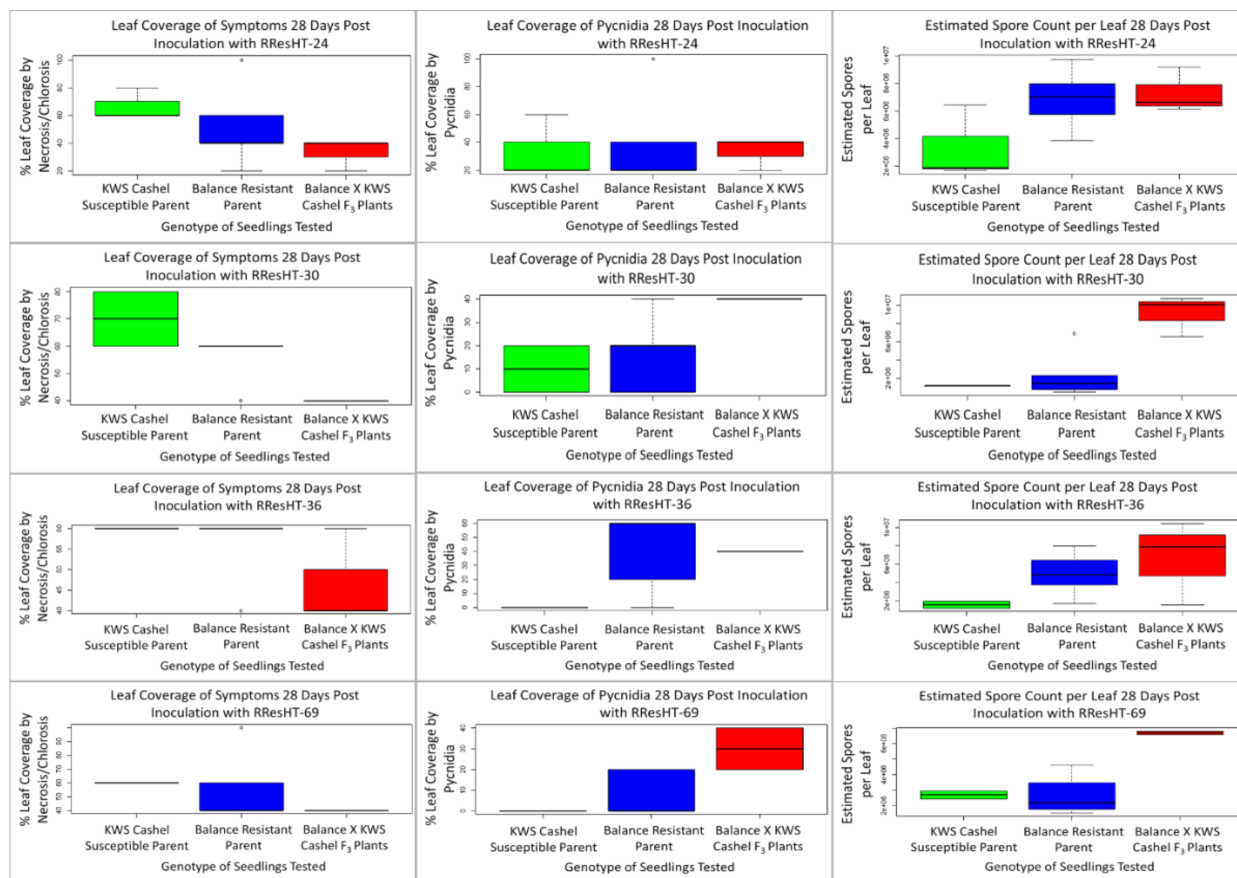

Figure 2: Phenotypes from Balance X KWS Cashel F_3 hybrids when tested against four *Z. tritici* isolates. Genotyping identified few reliable markers, likely due to low numbers of plants tested (data not shown). Green boxes represent the susceptible parental line, blue boxes represent the resistant parental line and red boxes represent F_3 hybrid plants.

Synthetic M3 x KWS Cashel F3 mapping population analysis

The F_3 segregating population produced from crossing the resistance source Synthetic M3 with KWS Cashel was used to test KASP markers associated with the *Stb* resistance genes *Stb16q* and *Stb17*. *Stb16q* has been cloned and extensively studied by the research community (Saintenac *et al.*, 2021). *Stb17* has also been studied, but has yet to be cloned. This test compared 34 F_3 hybrid plants, 16 Synthetic M3 resistant parental plants and 34 KWS Cashel susceptible parental plants. Known markers for *Stb16q* were confirmed to correctly predict the Synthetic M3 resistances to necrosis and pycnidia formation, but KASP markers near the location of *Stb17* did not. This may indicate that *Stb16q* confers a higher proportion of the Synthetic M3 resistance to the *Z. tritici* isolate used then *Stb17*. Alternatively, this could indicate that the marker used in this test is not adequately closely linked to *Stb17* to follow this gene through the segregating population, and does not remain linked to the resistance gene as it is inherited. This is always a possibility when markers do not adequately follow resistances, although it seems less likely to be the primary cause here as *Stb17* is known to be less effective in seedlings (Tabib Ghaffary *et al.*, 2012). The results for these markers are shown in Table 6.

Kavkaz-K4500 x KWS Cashel F3 mapping population analysis

The F_3 segregating population produced from crossing the resistance source Kavkaz-K4500 with KWS Cashel was used to test KASP markers associated with the *Stb* resistance genes *Stb6, Stb7, Stb10* and *Stb12.* This test compared 40 F_3 hybrid plants, 23 Kavkaz-K4500 resistant parental plants and 34 KWS Cashel susceptible parental plants. Known KASP markers for *Stb10* predicted the Kavkaz-K4500 resistance well for most symptoms. Markers tested for *Stb6, Stb7* and *Stb12* were not significantly associated with resistance to any measured symptom. This likely indicates that a large proportion of the Kavkaz-K4500 resistance to the *Z. tritici* isolate used is derived from *Stb10,* or that the genetic distance between the resistance genes *Stb6, Stb7* and *Stb12* and the markers for them was too great for the two to reliably segregate together. The results for these markers are shown in Table 7.

Table 5: The probability of a correlation between each KASP marker tested and phenotype measured in the TE9111 x KWS Cashel F_3 mapping population. No cells show any statistically significant correlation.

TE9111			*P*-Values				
Name of KASP Marker Tested	**Position of KASP Marker**	Expected Linked *Stb* Gene	Days to Symptom Development	Days to Pycnidia Development	Final % Symptoms Coverage	Final % Pycnidia Coverage	Spore Count
AX-94640607_k	Position 57437751, Chromosome 1B	*Stb11*	**0.298**	**0.833**	**0.627**	**0.753**	**0.405**

Table 6: The probability of a correlation between each KASP marker tested and phenotype measured in the Synthetic M3 x KWS Cashel F_3 mapping population. Green cells show a significant correlation ($P \leq 0.05$, ANOVA test).

Synthetic M3			*P*-Values				
Name of KASP Marker Tested	**Position of KASP Marker**	Expected Linked *Stb* Gene	Days to Symptom Development	Days to Pycnidia Development	Final % Symptoms Coverage	Final % Pycnidia Coverage	Spore Count
AX-94937231_k	Position 531239743, Chromosome 5A	*Stb17*	**0.698**	**0.262**	**0.105**	**0.378**	**0.493**
AX-94447473_k	Position 529808043, Chromosome 5A	*Stb17*	**0.973**	**0.143**	**0.105**	**0.478**	**0.463**
cfn80031	Position 590041499, Chromosome 3D	*Stb16q*	**0.000**	**-**	**0.00**	**0.00**	**0.086**
cfn80044	Position 590041493, Chromosome 3D	*Stb16q*	**0.000**	**-**	**0.00**	**0.01**	**0.097**

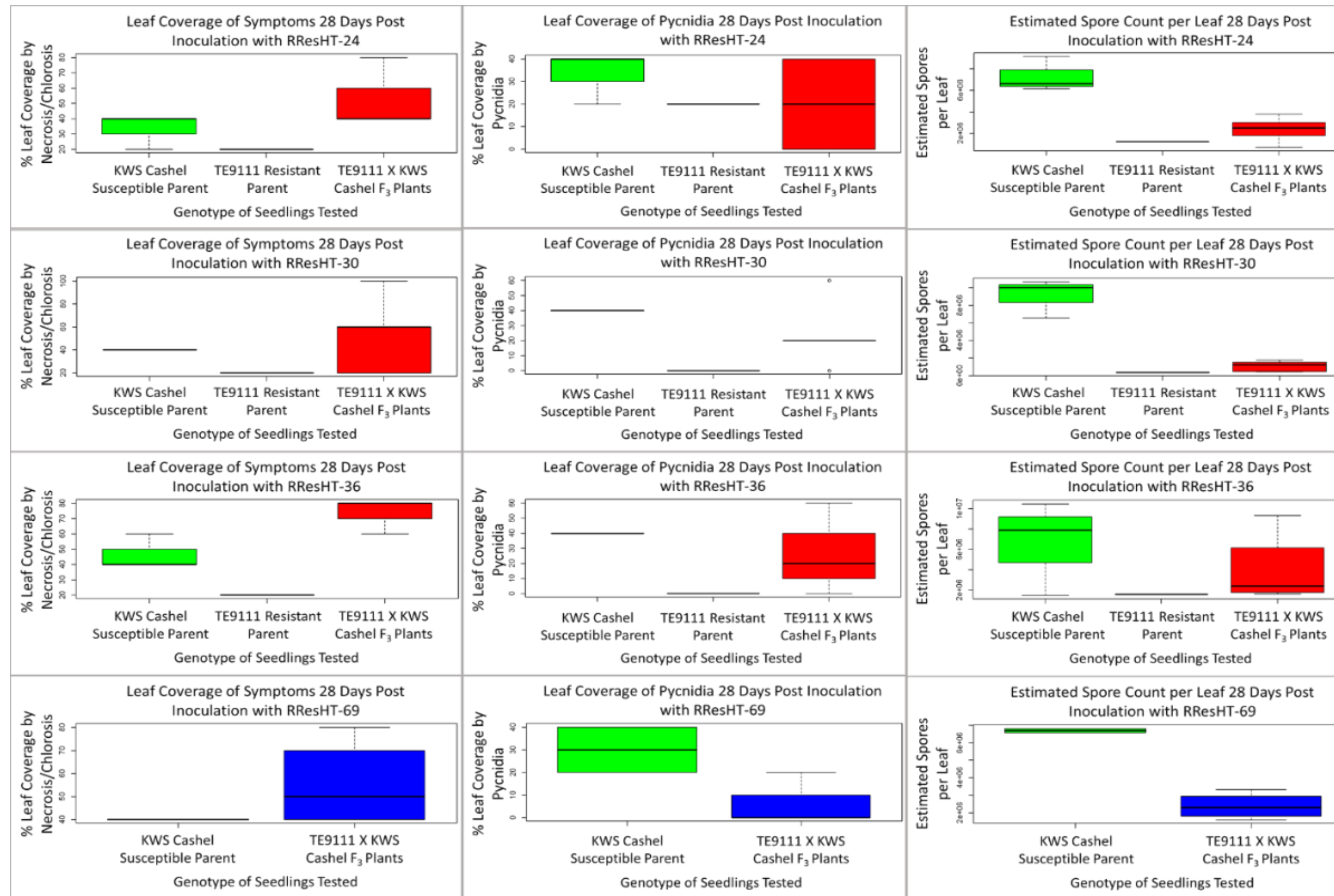

Figure 3: Phenotypes from TE9111 X KWS Cashel hybrids when tested against four *Z. tritici* isolates. Green boxes represent the susceptible parental line, blue boxes represent the resistant parental line and red boxes represent F_3 hybrid plants.

Salamouni x KWS Cashel F3 mapping population analysis

The F_3 segregating population produced from crossing the resistance source Salamouni with KWS Cashel was used to test KASP markers associated with the *Stb* resistance genes *Stb6, Stb13* and *Stb14*. This test compared 67 hybrid F_3 plants, ten Salamouni resistant parents and 16 KWS Cashel susceptible parents. Overall, none of the markers used for any of these genes showed significant correlation to either the time required for pycnidia development or the final percentage leaf coverage by pycnidia. This is likely due to the fact that pycnidia levels were generally low throughout the Salamouni F_3 segregating population, leading to little differentiation in these symptoms. The strongest correlations seem to be for the markers used for *Stb13*, two of which show significant correlations to both final % symptom coverage and final spore counts. This could suggest that the levels of necrosis that *Z. tritici* causes, and hence the fungal nutrition available for spore production, are significantly influenced by this resistance gene. The markers for *Stb14* showed little correlation with any symptoms of interest, although two (both designed for position 821089277, near one previous *Stb14* marker) did have some correlation to the final % symptom coverage. Unexpectedly the markers for *Stb6* showed some correlation to the days to symptom development and a small amount of correlation to the final spore count values, despite the fact that all *Z. tritici* isolates used in this project had previously been listed as virulent against *Stb6,* possibly indicating that *Stb6* (or a linked gene) still serves to increase the resistance or tolerance of this line to a greater effect than was initially assumed. However, the *P*-values for spore count here are close to the significance threshold of $P = 0.05$ and rate of symptom development is considered a low-impact symptom in this study. The results for these markers are shown in Table 8.

Bulgaria 88 x KWS Cashel F3 mapping population analysis

The F_3 segregating population produced from crossing resistance donor Bulgaria 88 with KWS Cashel was used to test KASP markers associated with the *Stb* resistance gene *Stb6*. Attempts were also made to test for associations using *Stb1* KASP markers, but the markers produced for this gene proved ineffective. This test compared 67 hybrid F_3 plants, nine Bulgaria 88 resistance donor parental plants and 16 KWS Cashel susceptible parental plants. As expected, none of the markers used for *Stb6* showed any significant correlation to any symptom measured. It would be beneficial to examine other sources of wheat genetic information to identify alternative SNPs for which *Stb1* KASP markers could be designed. The results for these markers are shown in Table 9.

Near Isogenic Lines (NILs) population analysis

Five NIL sets, produced by backcrossing the resistance donors Synthetic 6X, Kavkaz-K4500, Synthetic M3, Tadinia and Estanzuela Federal to susceptible KWS Cashel to the BC_3 generation, using KASP markers to ensure that the desired *Stb* genes were present/absent in each genotype, were tested for *Z. tritici* resistance. For each resistance source, two NIL lines were used, one of which was predicted (based on the KASP marker analysis) to contain the *Stb* genes of interest, the other of which was predicted to lack them. The Synthetic 6X-derived NILs were *Stb5* and *stb5-*, the Kavkaz-K4500-derived NILs were *Stb7/stb10/Stb12* and *stb7/stb10/stb12-*, the Synthetic M3-derived NILs were *Stb16q/stb17-* and *stb16q/stb17,* the Tadinia-derived lines were *Stb4* and *stb4-* and the Estanzuela Federal-derived lines were *Stb7* and *stb7-*. As Figures 4 and 5 show, these NIL pairs (and parental controls) were challenged with five different *Z. tritici* isolates.

The most thorough testing of the Synthetic 6X, Kavkaz-K4500 and Synthetic M3-derived lines was conducted with RResHT-4, using 283 BC_3 plants in total. Additional testing used fewer plants (approx. 12-20 BC_3 per line/isolate interaction) and was used to confirm that the trends seen in the bioassay results involving RResHT-4 also applied to those involving other *Z. tritici* isolates. The results show that *Stb5* and *Stb16q* are consistently capable of greatly reducing or preventing the development of disease symptom phenotypes (especially pycnidia development) in parental and resistant NIL lines. Slightly higher resistance in parental lines possibly indicates the presence of unknown QTLs that add to the resistance of these resistant wheat lines, although data from (Saintenac *et al.*, 2021) suggests that the correct haplotype for the *Stb16q* gene still provides total resistance to the 11 *Z. tritici* IPO isolates tested even when cloned into the susceptible cultivar Courtot. The Kavkaz-K4500-derived NIL lines (containing *Stb7* and *Stb12*) showed more variable resistance, suggesting that these *Stb* genes are less effective against some isolates, although the resistant parental line remained highly resistant. This could indicate that *Stb10* (not included in NIL lines due to a lack of usable linked markers during breeding) provides the remaining resistance in Kavkaz-K4500, or that all three *Stb* genes (and potentially further as yet unidentified resistance QTLs) operate together to provide robust disease resistance. Figures 4 and 5 show the range of symptoms measured in the Kavkaz-K4500, Synthetic 6X and Synthetic M3 derived NIL lines.

The Estanzuela Federal-derived lines were tested with the *Z. tritici* isolate RResHT-24, involving a total of 90 plants. The Tadinia-derived lines were tested with the *Z. tritici* isolate RResHT-34, involving a total of 99 plants. Both sets of results show that the positive and negative NIL lines have similar averages, in between the values found for their

respective parental lines (although this effect is clearer for the Estanzuela Federal-derived lines).

For the Estanzuela Federal-derived lines, the NIL line lacking any known resistance gene (the NIL- line) shows significantly different levels of pycnidia coverage from both parental lines in a single factor ANOVA test. Both NIL genotypes show significant differences in the estimated spore counts from the Estanzuela Federal parent. There is no significant difference between the two NIL genotypes (*Stb7+/stb7-*) for either symptom. This suggests that *Stb7* is not the primary resistance gene in Estanzuela Federal effective against the *Z. tritici* isolate used here, as both NILs appear to possess a partial resistance compared to the KWS Cashel susceptible parent (although this does not appear to be as effective as the full Estanzuela Federal resistance, potentially indicating the presence of multiple resistance QTLs in this line).

For the Tadinia-derived lines, both NILs have significantly different levels of pycnidia coverage than the resistant parent, and all lines are significantly different from one another regarding the spore count estimates. These results may suggest that the *Stb4* gene provides only some of the resistance of the Tadinia line, with *Stb4* providing no resistance affecting final levels of pycnidia coverage in NIL lines. Figures 6 and 7 show the range of pycnidia coverage and spore counts for the Tadinia and Estanzuela Federal-derived NIL lines.

Discussion

The development of modern genetic markers for both widely used and novel *Stb* resistance genes will be a vital part of generating stable resistance to Septoria tritici blotch in order to protect UK and European wheat from new virulent and fungicide-resistant *Z. tritici* strains. This objective has historically been important to protect European agriculture, but is likely to take on an additional urgency over the coming years as ongoing conflicts in Eastern Europe disrupt significant proportions of world wheat production, causing supply shortages and price raises.

Table 7: The probability of a correlation between each KASP marker tested and phenotype measured in the Kavkaz-K4500 x KWS Cashel F_3 mapping population. Green cells show a significant correlation ($P \leq 0.05$, ANOVA test).

Kavkaz-K4500			*P*-Values				
Name of KASP Marker Tested	**Position of KASP Marker**	Expected Linked *Stb* Gene	Days to Symptom Development	Days to Pycnidia Development	Final % Symptoms Coverage	Final % Pycnidia Coverage	Spore Count
AX-95175098_K	Position 740626510, Chromosome 4A	*Stb7*	**0.447**	**0.472**	**0.733**	**0.182**	**0.989**
AX-94444583_k	Position 34619199, Chromosome 1D	*Stb10*	**0.000**	**0.000**	**0.053**	**0.000**	**0.000**
AX-94798786_k	Position 34619771, Chromosome 1D	*Stb10*	**0.000**	**0.000**	**0.146**	**0.000**	**0.000**
AX-94645442_k	Position 733706257, Chromosome 4A	*Stb12*	**0.665**	**0.324**	**0.874**	**0.135**	**0.813**
AX-94531033_k	Position 735006493, Chromosome 4A	*Stb12*	**0.460**	**0.077**	**0.755**	**0.553**	**0.437**
AX-94863246_k	Position 732512417, Chromosome 4A	*Stb12*	**0.905**	**0.463**	**0.733**	**0.102**	**0.804**
cfn80025	Position 26136440, Chromosome 3A	*Stb6*	**0.970**	**0.307**	**0.331**	**0.077**	**0.619**
cfn80023	Position 26206170, Chromosome 3A	*Stb6*	**-**	**-**	**-**	**-**	**-**
cfn80030	Position 26234019, Chromosome 3A	*Stb6*	**0.635**	**0.280**	**0.597**	**0.503**	**0.672**

Table 8: The probability of a correlation between each KASP marker tested and phenotype measured in the Salamouni x KWS Cashel F_3 mapping population. Green cells show a significant correlation ($P \leq 0.05$, ANOVA test).

Salamouni			*P*-Values				
Name of KASP Marker Tested	**Position of KASP Marker**	Expected Linked *Stb* Gene	Days to Symptom Development	Days to Pycnidia Development	Final % Symptoms Coverage	Final % Pycnidia Coverage	Spore Count
AX-94893368_k	Position 540062500, Chromosome 7B	*Stb13*	**0.712**	**0.493**	**0.019**	**0.680**	**0.008**
AX-94395385_k	Position 539786477, Chromosome 7B	*Stb13*	**0.991**	**0.406**	**0.062**	**0.856**	**0.034**
AX-95202159_k	Position 548165329, Chromosome 7B	*Stb13*	**0.819**	**0.309**	**0.038**	**0.954**	**0.018**
AX-94791594_k	Position 23600083, Chromosome 3B	*Stb14*	**0.631**	**0.585**	**0.452**	**0.267**	**0.189**
AX-94591165_k	Position 23364024, Chromosome 3B	*Stb14*	**0.685**	**0.847**	**0.266**	**0.223**	**0.173**
AX-94790051_k	Position 821089277, Chromosome 3B	*Stb14*	**0.360**	**0.669**	**0.008**	**0.236**	**0.054**
AX-94790051_k	Position 821089277, Chromosome 3B	*Stb14*	**0.712**	**0.805**	**0.044**	**0.092**	**0.113**
cfn80025	Position 26136440, Chromosome 3A	*Stb6*	**0.019**	**0.061**	**0.127**	**0.206**	**0.041**
cfn80023	Position 26206170, Chromosome 3A	*Stb6*	**0.019**	**0.061**	**0.127**	**0.206**	**0.041**
cfn80030	Position 26234019, Chromosome 3A	*Stb6*	**0.017**	**0.183**	**0.070**	**0.659**	**0.062**

Table 9: The probability of a correlation between each KASP marker tested and phenotype measured in the Bulgaria x KWS Cashel F_3 mapping population. No significant correlation was found.

Bulgaria 88			*P*-Values				
Name of KASP Marker Tested	**Position of KASP Marker**	Expected Linked *Stb* Gene	Days to Symptom Development	Days to Pycnidia Development	Final % Symptoms Coverage	Final % Pycnidia Coverage	Spore Count
cfn80025	Position 26136440, Chromosome 3A	*Stb6*	**0.703**	**0.579**	**0.281**	**0.887**	**0.379**
cfn80023	Position 26206170, Chromosome 3A	*Stb6*	**0.703**	**0.579**	**0.281**	**0.887**	**0.379**
cfn80030	Position 26234019, Chromosome 3A	*Stb6*	**0.352**	**0.722**	**0.266**	**0.711**	**0.164**

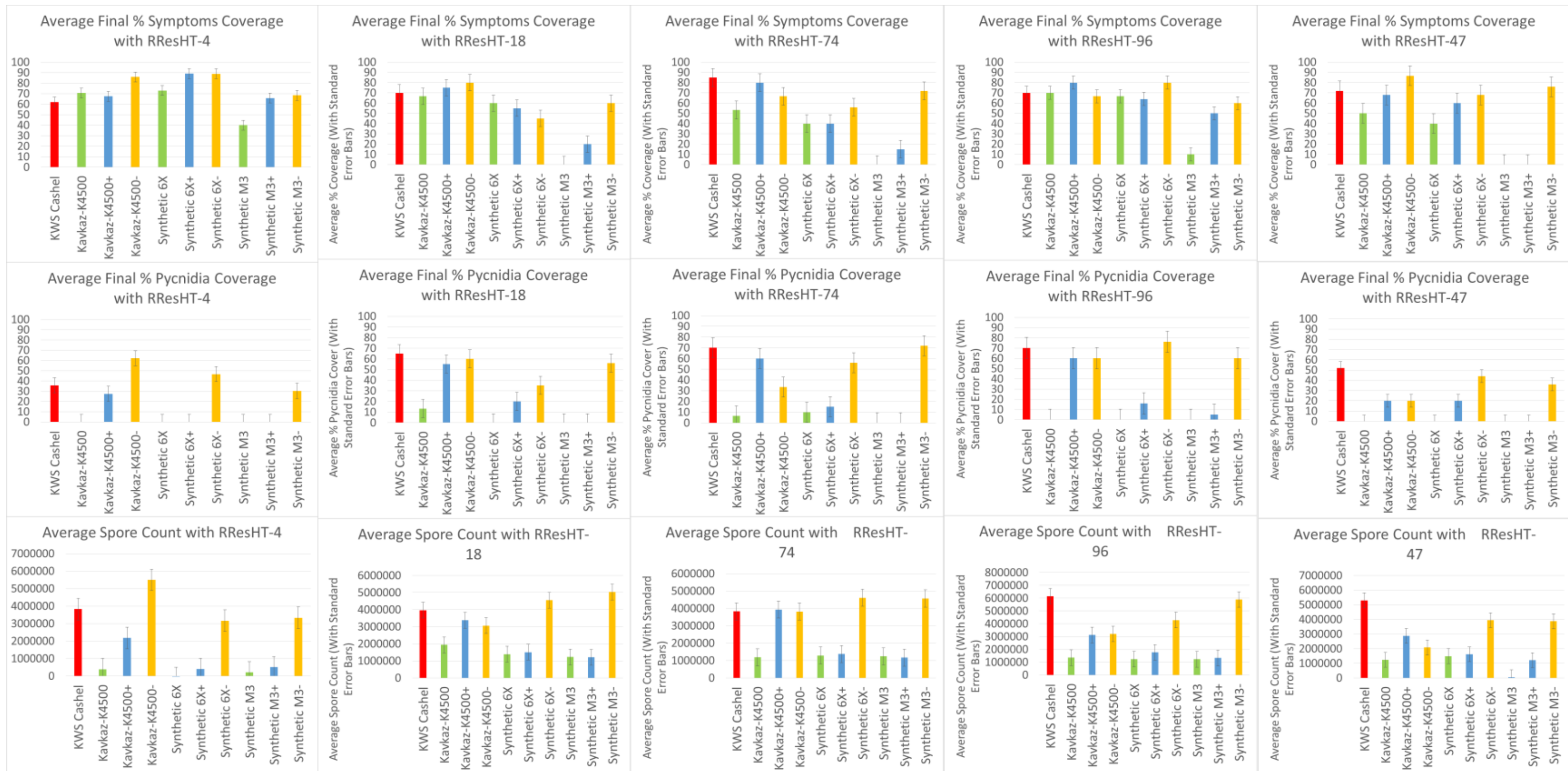

Figure 4: Graphs showing the symptom and pycnidia coverages and final spore counts of the tested Synthetic 6X, Kavkaz-K4500 and Synthetic M3-derived NIL lines against each *Z. tritici* isolate. Symptom and pycnidia development rates show less differentiation and are not included. The results from susceptible KWS Cashel control plants are shown with red bars, results from resistant parental control plants are shown with green bars, results from expected resistant NIL line plants are shown with blue bars, and results from expected susceptible NIL line plants are shown with yellow bars.

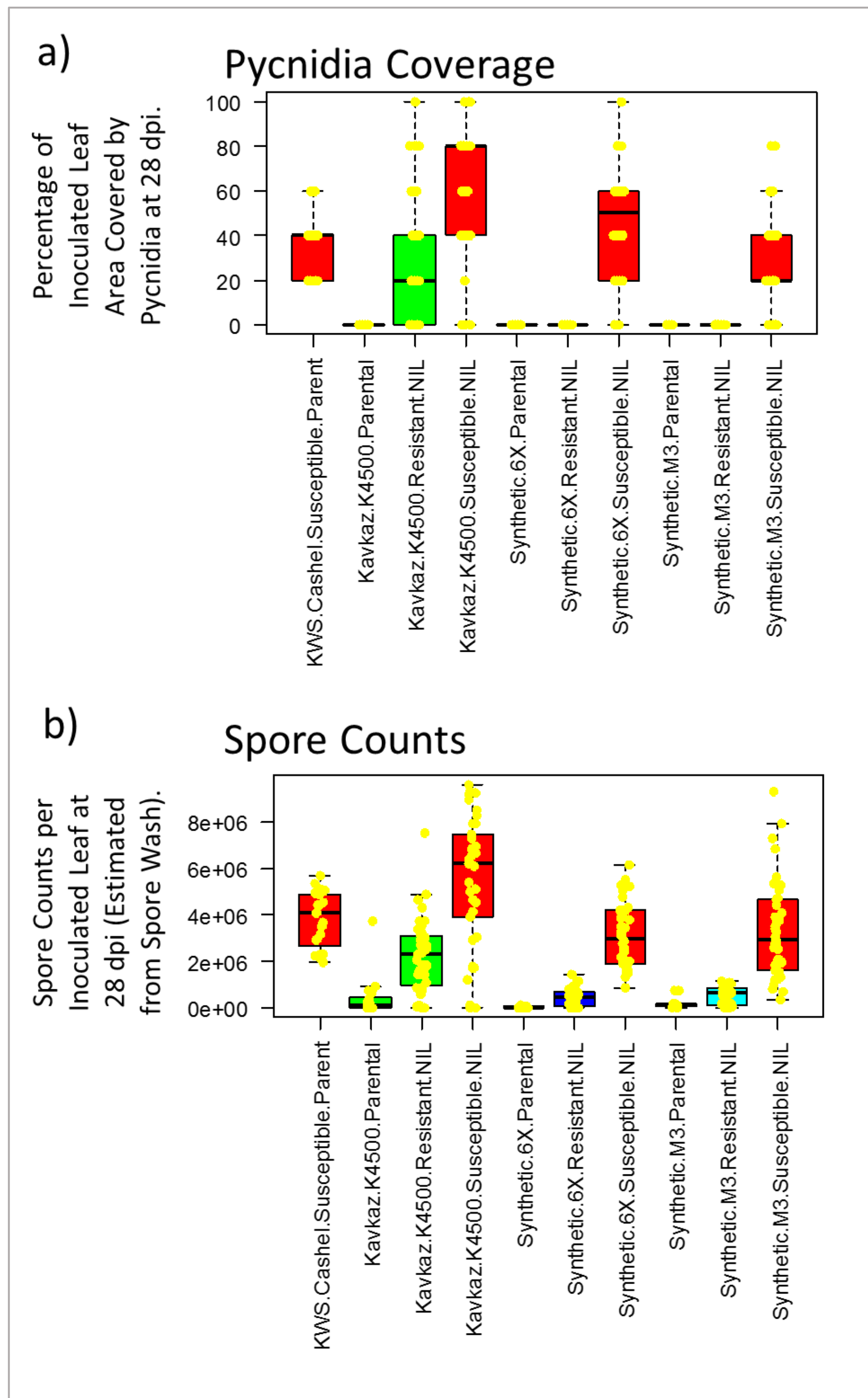

Figure 5: A boxplot showing the range of a) pycnidia coverages and b) spore counts for the Kavkaz-K4500, Synthetic 6X and Synthetic M3-derived NIL and parental lines. Red colourings indicate expected susceptible lines, whereas green and blue boxes indicate expected resistant lines (colour coded for expected resistance genes).

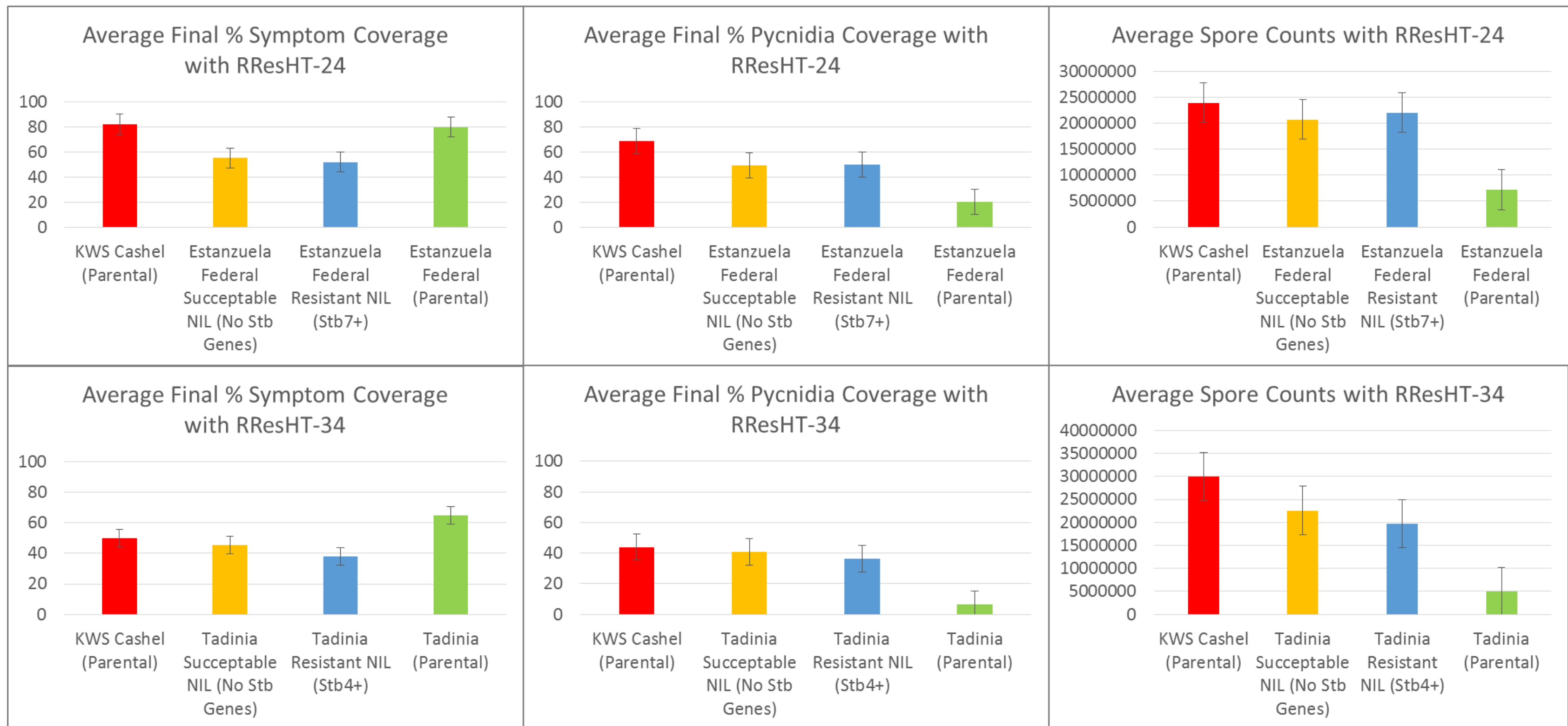

Figure 6: Graphs showing the symptom and pycnidia coverages and final spore counts of the tested Tadinia- and Estanzuela Federal-derived NIL lines against each *Z. tritici* isolate. Symptom and pycnidia development rates show less differentiation, and so are not included. The results from susceptible KWS Cashel control plants are shown with red bars, results from resistant parental control plants are shown with green bars, results from expected resistant NIL line plants are shown with blue bars, and results from expected susceptible NIL line plants are shown with yellow bars

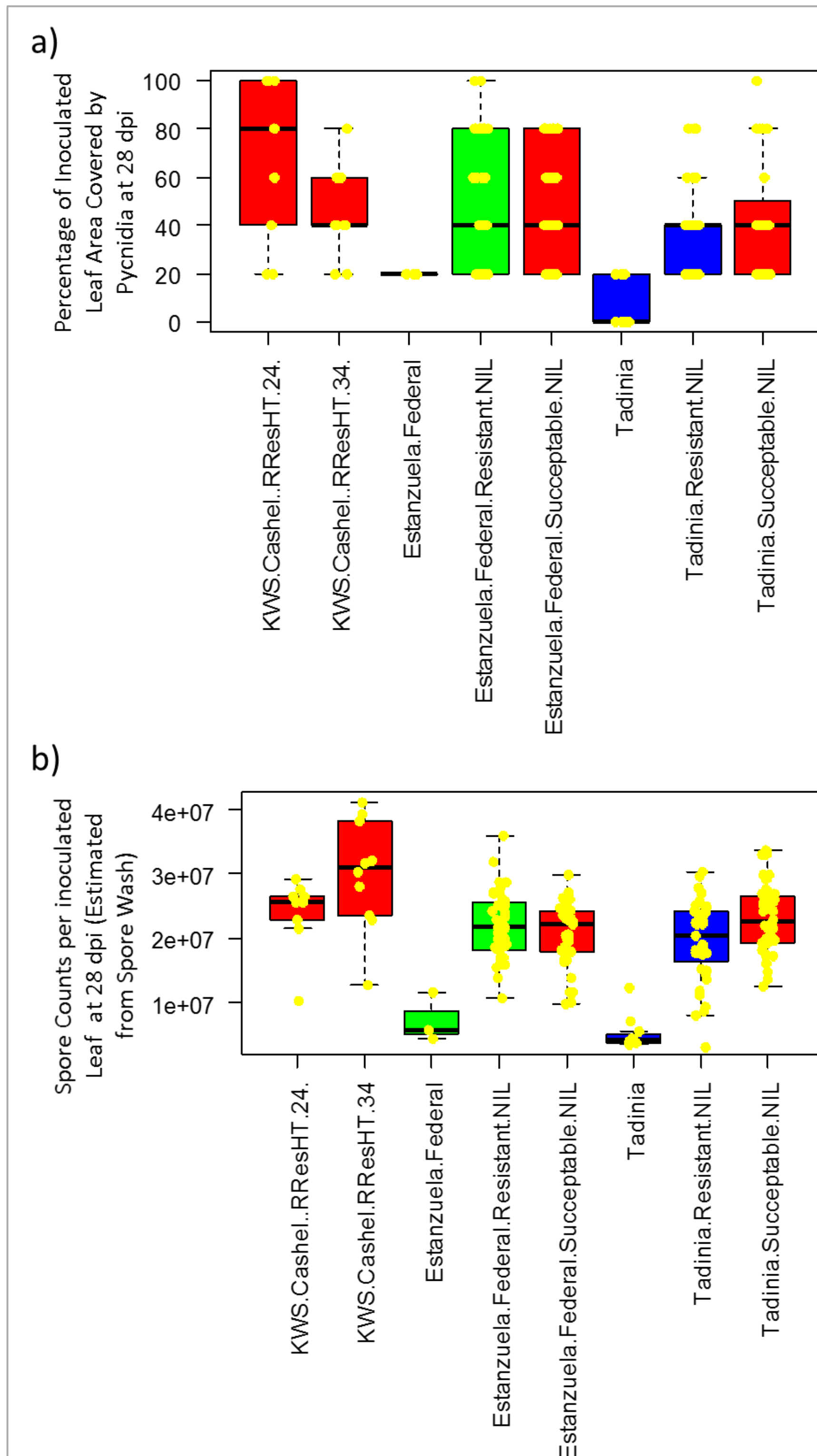

Figure 7: A boxplot showing the range of a) pycnidia coverages and b) spore counts for the Tadinia and Estanzuela Federal-derived NIL lines and their parental lines. Red colourings indicate expected susceptible lines, whereas green and blue boxes indicate expected resistant lines (colour coded for expected resistance genes).

The research presented here has helped to confirm and develop KASP markers for both *Stb* genes in existing use (e.g. *Stb18,* which is known to exist in the cultivars Apache and Balance (Tabib Ghaffary *et al.*, 2011)) and novel *Stb* genes (e.g. *Stb5*, derived from the synthetic line Synthetic 6X (Arraiano *et al.*, 2001)).

Unfortunately it was not possible to ensure that these markers were placed within the coding regions of each *Stb* resistance gene of interest in this study, due to the lack of available information related to these genes at the genetic level. This means that we cannot fully discount the possibility that some of these markers could become disconnected from the genes for which they were constructed through crossing over events in a large, long term breeding program (although such cases should be rare as successful markers should be physically close in the genome to the genes for which they act as markers). Obtaining more information on the exact genomic locations of *Stb* resistance genes would help to more accurately determine the risks of this occurring, as could performing large, long term screens in which the predictions of these markers were compared to crop resistance phenotypes across several generations. Additionally, although efforts were undertaken to identify unique SNPs were used as the basis for these markers wherever possible, due to the dearth of SNPs near the expected locations of some *Stb* resistance genes and the fact that not all wheat lines have been genetically sequenced, it is not currently possible to guarantee that all of these markers are completely unique to resistant lines. Therefore it would be advisable for breeders to test these markers on all relevant parental lines before using them in new breeding programs to ensure that the chosen markers differentiate as expected between all lines used. This is especially true for breeders using novel germplasm not included in this study. Nevertheless, these markers should, in most cases, provide a valuable tool for following high value, and in some cases relatively novel, *Stb* resistance genes in breeding populations.

Stb5 in particular has been recently shown to have a highly significant protective effect in the field in the UK (Ruth Bryant, personal communication, 2022). These high-throughput markers will aid in the breeding of new wheat lines for Septoria resistance by enabling large numbers of seedlings in each generation to be screened for the presence of novel *Stb* resistance genes of interest. This will allow much larger and faster breeding generations than would be feasible using traditional markers (e.g. SSR markers), which take longer to run for each seedling. KASP marker selection holds even greater advantages over attempting to track resistance genes by monitoring STB infection symptoms in the field, which may have otherwise been required for some of the more novel *Stb* resistance genes (for which reliable KASP markers are often not widely available due their relative newness, a

lack of interest from breeders who feel that the linkage drag often associated with such genes makes them unappealing economic prospects, and/or to a reluctance to share such information between competing breeding companies), as such phenotypic monitoring requires plants to be grown to maturity before testing can begin.

Some resistance genes are known to operate more efficiently at different stages of the host plants life cycle (possibly including those tested here, although other resistances used, such as *Stb5,* are known to operate at both seedling and adult plants stages (Arraiano *et al.*, 2001)). However, KASP markers are able to identify the presence of such genes at any point of the wheat plants life cycle. It is possible that some of the *Stb* genes considered here will be less useful to breeders if their effectiveness is reduced in adult plants, but in these cases it is still likely that some measure of useful protection will be provided at some point in the life cycle. KASP markers help to enable the use of other new techniques to increase the rate of the breeding process. For example, single seed decent can operate considerably more effectively with high-throughput markers, which allow the selection of specific genes of interest to be conducted alongside more traditional screening, speeding up the breeding process and increasing the number of properties that can be bred for simultaneously. Effective markers also make it possible to track multiple resistances simultaneously in a single line without the need for testing with multiple *Z. tritici* isolates collectively containing a selection of relevant avirulence genes.

Effective markers for the resistance genes *Stb7* and *Stb11* were not found during this experiment. In the case of *Stb7*, this is assumed to be due to the weakness of the *Stb7* resistance compared to other, currently unknown, resistances present in Estanzuela Federal, as well as a general lack of good differentiation between the disease symptoms of the resistant Estanzuela Federal and susceptible KWS Cashel parental genotypes used to produce the segregating population. In this case, it is possible that the development of further NIL lines could isolate the *Stb7* gene and permit easier study and the development of *Stb7* markers. Alternatively, it is possible that the markers used here for *Stb7* were not sufficiently closely linked to the resistance and thus were not able to follow it reliably enough to identify a significant link to symptom development from the sample size used here. In this case, identifying markers closer to the true location of *Stb7* may allow this resistance to be followed more effectively. In either case, identifying *Z. tritici* populations that allow better differentiation between the parental lines could also allow better markers for the *Stb7* gene to be produced, and may reveal statistically significant links between the current markers and resistance effects.

Further mapping studies using *Z. tritici* isolates virulent against *Stb7* could also allow the alternative resistance present in Estanzuela

Federal to be identified and used in breeding programs. Although the low resistance of Estanzuela Federal to most *Z. tritici* isolates suggests that both resistances drawn from this line are likely to be of low utility in UK wheat lines, one or both may be useful in wheat grown elsewhere, which may help to protect UK wheat indirectly by preventing virulent *Z. tritici* lines from developing against other, more broadly effective *Stb* genes in gene pyramids.

In the case of the resistance gene *Stb11*, it is likely due to the lack of suitable marker and SNP locations that no effective markers could be identified, as this gene appears to be responsible for a large portion of the broad and highly effective resistance of the TE9111 line. Only five testable *Stb11* markers could be identified, and of these only one proved able to differentiate between the KWS Cashel and TE9111 parental genotypes. This may indicate that *Stb11* is in an area of the genome where there are relatively few single nucleotide polymorphisms suitable for the targeting of KASP markers. However, the development of additional markers is still highly desirable for such a potentially valuable resistance gene. It would therefore be a useful aim for future work to produce new marker candidates from alternative sources of genetic information and test them for potential viability in breeding programs. Even non-unique markers useful only for differentiation in breeding programs containing specific wheat lines would be valuable for such an effective *Stb* resistance gene.

Overall, these results support previous work (Tabib Ghaffary *et al.*, 2012; Mahboubi *et al.*, 2022) indicating that novel resistance genes are likely to have high individual impacts in improving resistance to Septoria tritici blotch disease in wheat, thus indicating that identifying modern markers for novel *Stb* resistance genes should be a priority for breeders. More broadly, systematic testing of wheat landraces and synthetic lines to identify resistance-associated KASP markers and map the associated QTLs should be conducted. It is important that varied *Z. tritici* isolates are either tested against relevant parental lines prior to use or used directly to test segregating populations to account for the presence of novel resistances that do not act against all *Z. tritici* isolates. The work presented here has already identified potential unknown resistance QTLs in Estanzuela Federal, and possibly in Tadinia – further work to identify these QTLs should be undertaken so that markers for them can be identified.

The effective *Stb* genes identified in this and other work should be used responsibly by breeders, with due consideration for the long term efficacy of the associated resistance genes. The examples of Kavkaz-K4500 and TE9111, both of which retain effective resistance despite being breeding lines of significant age, suggests that the most effective way to do this may be through the formation of gene pyramids involving multiple resistance genes. This may be effective because *Z. tritici*

isolates would only become virulent against such plants if multiple mutations occurred simultaneously to enable a new *Z. tritici* strain to become virulent against all the resistances involved, which becomes exponentially less likely as more genes are added to the resistance pyramid. However, such a system would only work if all breeders and growers limited their use of novel resistances to deployment in such pyramids, as otherwise virulence's could be gained by *Z. tritici* isolates against individual *Stb* resistance genes present in isolation in otherwise unprotected wheat lines and then inherited by a single strain during *Z. tritici's* sexual reproduction cycle.

The KASP markers demonstrated to follow effective resistances in this study will form an important part of the efforts to incentivise this kind of responsible breeding structure amongst breeding companies, who must weigh the profitability of different breeding schemes when producing new lines. KASP markers are high throughput, relatively cheap and capable of tracking both homo- and heterozygous genetic states in very young seedlings. This makes it vastly more time and cost effective for breeders to follow several *Stb* genes through breeding programs, which would otherwise be extremely difficult due to the need to use several *Z. tritici* isolates to identify which resistances each plant in the program possessed (as an *Z. tritici* isolate that is avirulent on wheat plants containing one *Stb* resistance gene is not going to be useful for following any further *Stb* resistance genes in a line that already contains an *Stb* resistance gene to which that isolate is avirulent). Additional incentives to use the KASP markers this way instead of pursuing the lower investment, short-term gain associated with relying upon a single *Stb* gene may be required. Such incentives could include regulation by regional or national government bodies (e.g. DEFRA in the UK), or be organised through agreements within the breeding market based on the long term benefits for all the companies involved. In either case, involvement from the academic community will be required to argue the importance of these breeding limitations, and to monitor which *Stb* genes should be protected in gene pyramids and which already have sufficient virulence against them present in the *Z. tritici* population that this level of protection is no longer warranted for such widely broken *Stb* resistance genes.

Chapter 4 - The production and testing of fluorescent *Z. tritici* strains as research tools for the examination of *Stb* resistance gene mechanisms

Abstract

Although *Z. tritici* is an important and much studied pathogen, little is currently known about the defence mechanisms utilised by most of the *Stb* resistance genes available for its control in wheat germplasm. Recent studies have suggested that stomatal closure may be an important defence response triggered by some of the characterised *Stb* genes. This project details the production of fluorescent *Z. tritici* strains that can be used to examine *Z. tritici* – leaf surface interactions on resistant wheat lines in order to determine the extent to which each *Stb* resistance gene triggers stomatal closure as a defence response. The sequences of constructs that enabled the production of GFP and mCherry fluorophores in multiple *Z. tritici* strains are reported. Six *Z. tritici* isolates with useful virulence profiles for the examination of *Stb* resistance gene mechanisms were confirmed through stereomicroscopy and confocal microscopy to have been successfully transformed to produce such fluorescent strains. Two of these strains were used in a preliminary study utilising pairs of wheat NIL lines containing or lacking the *Stb5* resistance gene with the aim of refining the methodology for conducting such examinations using these fluorescent strains, and although the data captured here does not clearly show the expected trend of *Stb5* containing leaves having reduced proportions of open stomata when inoculated with avirulent *Z. tritici* strains, a number of adjustments are suggested for future work to ensure that more beneficial data can be captured using the tools developed and described here.

Contributions

My contributions to this chapter include:

- The growth, maintenance and where necessary purification of all *Z. tritici* isolates used.
- The design and preparation of constructs for *Z. tritici* transformation (using components donated by Maiara Piovesana and Carlos Bayon).
- The transformation of *Agrobacteria* and *Z. tritici* using these constructs.
- The growth and preparation of all host plants used.
- The inoculation of host plant leaves.
- The harvesting of leaves and their examination and photography under confocal microscopy at intervals throughout the infection process.
- The analysis and phenotyping of leaf surface images.
- The statistical analyses and interpretation of results.
- The writing of this chapter.

Other contributions to this chapter include:

- The initial provision of wild-type fungal isolates used by Bart Fraaije.
- Advice from my supervisory team (Kostya Kanyuka, Jason Rudd, Rumiana Ray and Ruth Bryant) throughout the experimental and writing processes.
- Practical advice and technique demonstrations from Carlos Bayon (*Z. tritici* transformations) and Kirstie Halsey (stereo- and confocal microscopy).

Introduction

Zymoseptoria tritici, the causal agent of Septoria tritici blotch (STB) disease, is one of the most economically important crop diseases in Europe, along with being an issue in North Africa and Australia (Fones and Gurr, 2015). Approximately 70% of the fungicides used in Europe is used to prevent STB epidemics, at an annual cost of around €1 billion (Duveiller *et al.*, 2007; Torriani *et al.*, 2015). This places a significant cost burden on wheat growers, and causes environmental damage which can weaken ecosystem services, e.g. poisoning predators can leave monocultures more vulnerable to other pests, such as the reduction of egg predation on the grapevine pest *Lobesia botrana* caused by increased frequencies of fungicide applications (Pennington *et al.*, 2018). These barriers to fungicide use make it more challenging to ramp up grain yields in Europe due to the potential for high yield losses.

This yield loss is going to be an increasing issue as demand for wheat products increases with increasing world populations and greater demand for food in currently underdeveloped countries. Additionally, climate change is likely to have a significant impact on cereal yield levels over much of the world, both directly (through abiotic environmental changes) and indirectly (through the changing ranges of numerous pests and diseases) (Yeo, 1998; Anderson *et al.*, 2004; Parry *et al.*, 2005; Wassmann *et al.*, 2009; Ramankutty, 2016). For example, increases in rainfall are likely to increase levels of *Z. tritici* infection, as the pathogen is spread largely through water droplet splashes in its asexual stage and demonstrates far higher infection success when leaves are kept damp for approximately three days following infection exposure. This is likely to create a need for growers to deal with *Z. tritici* epidemics in areas where STB has not historically been a high concern. Therefore, improving breeder’s and grower’s ability to mitigate STB damage to wheat crops should be a significant research priority.

Fungicide treatment applications are often expensive and time consuming for growers operating with relatively thin profit margins. Thus producing crops broadly and stably resistant to fungal diseases is

a more sustainable option for disease control. Historically, success has been had in other pathosystems (Singh *et al.*, 2011; Lillemo *et al.*, 2012; Kolmer, 2013; Ellis *et al.*, 2014; Singh *et al.*, 2016) using isolate-nonspecific resistance genes to build stable resistance to damaging diseases in plants. Sufficient broad spectrum resistances can render elite crop plant lines fully resistant to the disease from which it is being protected.

STB has traditionally been controlled with a limited number of chemical fungicides and wheat *Stb* resistance genes and QTLs. Unfortunately, the high genetic flexibility and sexual reproduction of *Z. tritici* has resulted in the emergence of *Z. tritici* isolates resistant to every major fungicide class currently in use against this disease, and in the emergence of virulent isolates against most of the widely used major resistance genes (Birr *et al.*, 2021).

The loss of effectiveness of major resistance genes over time has created a need for new resistance genes in wheat breeding programmes. Over 20 *Stb* resistance genes are currently known, some of which have not been widely used in wheat breeding (generally those identified in landraces and/or synthetic wheat lines). With appropriate markers and pre-breeding programs to remove undesirable linked traits from such non-elite lines, these *Stb* genes could provide broad STB protection to modern wheat. Unfortunately, previous experience has shown that single *Stb* genes bred into elite lines are rapidly broken as *Z. tritici* strains emerge that are virulent against them (e.g. *Stb16q* was recently introduced to elite wheat lines, but has already been broken in Ireland, Iran and France (Dalvand *et al.*, 2018; Kildea *et al.*, 2020)). It is therefore desirable to combine multiple *Stb* resistance genes in gene pyramids, which seem to be more durable on average (e.g. Kavkaz-K4500 contains a gene pyramid of *Stb6, Stb7, Stb10* and *Stb12* which has been used as a resistance source for decades (Kema and Da-Zhao, 1993; Kema *et al.*, 1996; Chartrain, Berry, *et al.*, 2005; Chartrain, Brading, *et al.*, 2005)), likely because each *Z. tritici* isolate is less likely to simultaneously develop the multiple mutations necessary to overcome such multi-gene resistance (Chartrain, Berry, *et al.*, 2005).

The *Stb* gene pyramiding process may be more efficient if the resistance genes included operate through different resistance mechanisms, as this would minimise the chance of a single mutation that produced a *Z. tritici* isolate capable of avoiding or neutralising a single such mechanism generating virulence against multiple resistance genes. Unfortunately, little is currently known regarding the mechanisms of most *Stb* genes, and the lack of data available on their genetic identities makes it difficult to examine such mechanisms through traditional knockout or knockdown studies.

Stb6 and *Stb16q* are the only *Stb* resistance genes that have so far been cloned (Saintenac *et al.*, 2018; Saintenac *et al.*, 2021). *Stb6*, *Stb9* and *Stb16q* were also tested in Battache *et al.*, 2022, which tested potential mechanisms by which these resistances could operate and suggested a stomatal defence component. The avirulence factors associated with these defence genes have only been cloned for *Stb6* (Zhong *et al.,* 2017) and *Stb9* (Amezrou *et al.*, 2022), named *AvrStb6* and *AvrStb9* respectively. Therefore *Stb6, Stb9* and *Stb16q* are the resistance genes we have the most molecular and mechanistic information on, and consequently are in the best position to further examine the resistance mechanism.

Battache *et al.* (2022) demonstrated that a significant element in the *Stb16q* resistance seems to be the closing of leaf stomata in the early pre-penetration stages of *Z. tritici* infection, resulting in 95% of penetration attempts being unsuccessful in an incompatible interaction. Results from fungal spore infiltrations into *Stb6* and *Stb9* containing lines also suggested that leaf surface penetration may be a significant barrier to successful infection in incompatible interactions involving these resistance genes as well (as infection levels were significantly higher when the leaf surface was bypassed through wounding). This system appears to operate on the principle that fewer hyphae penetrating the leaf surface will result in the formation of proportionally fewer pycnidia at the end of the infection process, as there are fewer opportunities for the necessary growth and spread of fungal infection. However, the remaining 5% of successful penetration suggests that additional mechanisms must be present to prevent infection spread in the interior of the leaf. This additional mechanism is not currently well understood.

Stb6 has been shown to be a wall-associated kinase (WAK) gene (Saintenac *et al.*, 2018), recognising the rapidly evolving avirulence factor *AvrStb6* (Zhong *et al.*, 2017). As *Stb16q* is a plasma membrane cysteine-rich receptor like kinase, this suggests that both known immune receptors providing resistance against *Z. tritici* may operate on the outer surface of plant cells. This would fit with a situation in which guard cells containing cell surface receptors generally identify avirulent *Z. tritici* isolates and close the stomata before penetration can occur.

Other pathosystems have also established stomatal closure as a viable and likely ancestral mechanism of plant defence. In particular, the model plant Arabidopsis has been demonstrated to respond to bacterial infection in part with a reduction in stomatal aperture and the thickening of cell walls near the leaf surface. Biochemical mechanisms have been suggested for this effect in the Arabidopsis system, e.g. via the protein AtGAP1 (Cheng *et al.*, 2022). It has also been suggested that relative air humidity can influence the effectiveness of this defence (Panchal *et al.*, 2016), indicating that this defence system is likely to act

by influencing stomatal regulatory networks, rather than only being a symptom of disease-related damage.

The screens required to determine whether similar mechanisms of resistance are used in the *Z. tritici* pathosystem would require NIL lines for each *Stb* resistance gene for which the mechanism was to be tested, along with a library of *Z. tritici* isolates containing both virulent and avirulent isolates against each included *Stb* resistance gene. This project focused on producing these resources and developing preliminary experiments to test the level of efficacy at which *Stb* genes operated in the NIL lines, and the impact of co-inoculating virulent and avirulent *Z. tritici* onto these lines.

The hypotheses of this research are: (1) that fluorescent *Z. tritici* strains can be produced from isolates identified in Tidd *et al.*, 2023 to be virulent or avirulent against wheat lines containing *Stb* resistances of interest, using the transformation techniques described in Motteram *et al.* (2009); (2) that these GFP- and mCherry-expressing transformed *Z. tritici* strains will possess the same virulence profiles as the wild type ancestors from which they were derived; and (3) that the *Stb5* resistance gene operates at least in part by triggering stomatal closure in response to *Z. tritici* infection. The aims of this project were to produce fluorescent *Z. tritici* strains that can be used for examining leaf surface interactions in the wheat-*Z. tritici* pathosystem, and to begin optimisation of experimental techniques that could be used to monitor *Z. tritici*-leaf surface interactions to determine whether stomatal closure was involved in resistance responses.

Materials and methods

Production of fluorescent protein expression constructs

Plasmid constructs were produced containing *green fluorescent protein* (*GFP*) and *mCherry* (*mCh*) fluorophore genes, under the control of a *Z. tritici* native *Tubulin* (*Tub2*) gene promoter and terminator. Components for these constructs were kindly donated by Maiara Piovesana (*GFP* and *mCh* sequences). These plasmids were constructed in the pCHYG vector kindly donated by Carlos Bayon. The sequences of the completed constructs are given in Supplementary Figure 2 of the Appendix.

The amplification of these components was carried out through PCR cloning. This primarily followed the recommended protocol following the NEB Gibson Assembly Cloning Kit Instruction manual. In this protocol, Q5 High-Fidelity DNA Polymerase (New England Biolabs) is used to amplify fragments using primers designed to produce overlaps during Gibson Assembly. These 30-40 bp long primers for this were designed using the NEBuilder online tool, overlapping on both sides

with expected PCR fragment sequences. The sequences of these primers are given in Supplementary Figure 3 of the Appendix. The final concentrations in the reaction mixtures used for these PCR reactions were 200 µM dNTPs, 0.5 µM of each primer, 0.02 U/µl of Q5 High-Fidelity DNA Polymerase and 0.1 µg/ml template DNA, in a mixture with 20% v/v 5X Q5 Reaction Buffer (New England Biolabs). This mixture was gently spun down to ensure all components were collected at the bottom of the tube, and then heated in a thermocycler. This process involved a 45 second initial denaturation stage at 98°C, followed by 38 cycles of 10 seconds at 98°C, 30 seconds at 56°C and 30 seconds at 72°C. This process was concluded with a 2 minute 30 second final extension stage at 72°C, after which the product was held at 4°C until collected and returned to storage at -20°C. Total volume for these reactions was routinely increased from the recommended 50 µl to 100 µl to ensure sufficient production of template DNA for the Gibson Assembly.

The PCR products produced then underwent DpnI digestion to remove unwanted plasmid template before the Gibson Assembly reaction. This process involved the production of a mixture containing an 8:1:1 v/v ratio of PCR product to 10X Cutsmart buffer (New England Biolabs) to 20 Units/µl DpnI enzyme. This mixture was then incubated for 30 minutes at 37°C before inactivation with a 20 minute incubation at 80°C.

This Gibson Assembly used the standard Gibson Assembly Master Mix (New England Biolabs) according to the manufacturer's instructions for a 4-6 fragment assembly with minor modifications. This protocol used a total fragment concentration 1 pmol/µl and a two-fold molar excess of vector in a 50% dilution of Gibson Assembly Master Mix (2X), made up to 60µl with nuclease free H_20. This mixture was incubated at 50°C in a thermocycler for 1 hour before being transferred to -20°C for longer term storage.

Gibson assembly products were transformed into chemically competent *E. coli* using heat shock. These *E. coli* were plated out on LB plates augmented with 50 µg/ml kanamycin and grown overnight at 37°C before selected colonies were tested by colony PCR (cPCR) using DreamTaq (ThermoFisher) according to the manufacturer's instructions with the forward primer for the *Tub2* promoter and the reverse primer for the *Tub2* terminator (i.e. flanking the entire ~3000 bp insert region).

Colonies that gave the correct band in cPCR reactions were then grown up overnight in LB broth (37°C, 220 rpm) and the plasmid construct was purified from them using a Qiagen Miniprep kit according to the manufacturer's instructions. The plasmid constructs were purified using a QIAquick PCR Purification Kit (Qiagen) according to the

manufacturer's instructions, then tested by double digest using *EcoR*I and *Hind*III restriction enzymes to verify the correct construct had been formed. Products of this digestion were then run on an electrophoresis gel and visualised using ethidium bromide. Correct plasmids were transformed into electro-competent Agrobacterium strain AGL1 by electroporation (Lin, 1995).

Transformation of *Z. tritici*

The *Z. tritici* isolates RResHT-1, 22, 42, 44, 45 and 52 were used for transformation. These isolates were selected primarily based on results from Tidd *et al.*, 2023 due to their virulence profiles on wheat lines of interest. RResHT-1 acts as a general avirulent control strain, avirulent on almost all wheat lines of interest except Balance (for which RResHT-22 will act as the avirulent strain). RResHT-42 is virulent on Tadinia, RResHT-44 is virulent on Balance, RResHT-45 is virulent on Balance and partially virulent on Synthetic 6X, and RResHT-52 provides the highest virulence of the available lines on Synthetic 6X. These virulence profiles were chosen to enable the examination of the mechanisms of the most broadly effective *Stb* resistance genes available in established wheat NIL populations accessible to researchers, through the production of virulent and avirulent *Z. tritici* strains against each *Stb* resistance gene of interest, with virulent and avirulent strains expressing different, differentiable fluorescence patterns in each case.

Although the general avirulent isolate RResHT-1 appeared to have lower aggressiveness on average than most of the *Z. tritici* isolates tested, and RRes-44, 45 and 52 all seem slightly more aggressive overall, the isolates used here were chosen primarily for their specific virulence profiles rather than for their average overall aggressiveness levels. Although isolates considered highly aggressive may in general be virulent against a broader spectrum of *Stb* resistance genes than isolates considered less aggressive, this does not necessarily imply a greater likelihood of virulence against novel resistance genes in a gene-for-gene resistance system, as this will be dependent on the presence or absence of the associated avirulence factor. The isolates used here and their virulence profiles are listed in Table 1.

Z. tritici transformation was accomplished using the methodology described in Motteram *et al.*, 2009. To carry out this method, the *Z. tritici* isolates to be transformed were grown on YPD agar for 5-6 days at 17°C in preparation for the transformation process. Transformed Agrobacterium strains containing the fluorescence protein expression constructs were grown on LB plates amended with kanamycin for selection for 4 days at 28°C in preparation for the transformation process. Small amounts of Agrobacterium stocks were applied to plates using a plastic loop to encourage the growth of single colonies.

One day before the transformation, one Agrobacterium colony from each selective plate was transferred to a 250 ml conical flask containing 40 ml LB Mannitol amended with two milligrams of kanamycin. This flask was incubated at 28°C, 220 rpm for 20-24 hours.

Table 1: The *Z. tritici* lines confirmed to have been successfully transformed to express fluorescent proteins, with their virulence profiles against wheat lines of interest. Grey cells indicate untested isolate-host combinations.

***Z. tritici* isolate**	**KWS Cashel**	**Synthetic 6X**	**Tadinia**	**Estanzuela Federal**	**Balance**
RResHT-1	Virulent	Avirulent	Avirulent	Avirulent	-
RResHT-22	Virulent	Virulent	Virulent	-	Avirulent
RResHT-42	Virulent	Partially virulent	Virulent	-	Virulent
RResHT-44	Virulent	-	Avirulent	Virulent	Virulent
RResHT-45	Virulent	Virulent	Partially virulent	Partially virulent	Virulent
RResHT-52	Virulent	Virulent	Virulent	Virulent	Partially virulent

Two millilitres of this media (increasing up to 20 ml as necessary if Agrobacterium growth was low) was centrifuged at 13 krpm for 2 min. The supernatant was discarded, and the pellet formed was resuspended in 400 µl induction medium (IM) amended with 0.04 mg/ml acetosyringone (IM:AS). This suspension was then centrifuged at 13 krpm for 2 min. The supernatant was discarded, and the pellet formed was resuspended in 1 ml IM amended with 0.04 mg/ml acetosyringone and 0.05 mg/ml kanamycin (IM:AS:Kan).

The 1ml Agrobacterium cell suspension was then supplemented with an additional 9 ml IM:AS:Kan and diluted to an optical density of 0.15 at 600 nm (OD_{600}). The resultant mixture was then incubated at 28°C, 220 rpm, and the OD_{600} was measured every 45 min. Once the Agrobacterium cells showed exponential growth with an OD_{600} of 0.19-0.26, they were removed and used to conduct the transformation.

Simultaneously, the blastospores of *Z. tritici* isolates to be transformed were harvested from agar cultures into 10 ml IM:AS media (adjusted according to level of fungal growth on the plate). The density of these blastospores was then measured using a haemocytometer and adjusted to approximately 1.6×10^7 spores/ml. This *Z. tritici* was then kept on ice until use.

Once both the *Z. tritici* and Agrobacterium were ready, 600 µl of each and 6 µl of 50 mg/ml of acetosyringone were mixed by flicking in a 1.5 ml Eppendorf tube. 200 µl of this mixture was then spread onto a cellulose membrane spread over an IM plate amended with 0.05 mg/ml acetosyringone. This plate was left unsealed and incubated at room temperature for 2 days.

After the incubation period, *Aspergillus nidulans* minimal medium agar plates were produced. This required 20x salts (120 g/L $NaNO_3$, 10.4 g/L KCl, 10.4 g/L $MgSO_4.7H_2O$ and 30.4 g/L KH_2PO_4 dissolved in sterile water) and trace elements (27.5 g/L $ZnSO_4.7H_2O$, 13.75 g/L H_3BO_3, 6.25 g/L $MnCl_2.4H_2O$, 6.25 g/L $FeSO_4.7H_2O$, 2 g/L $CoCl_2.5H_2O$, 2 g/L $CuSO_4.5H_2O$, 1.375 g/L $(NH_4)_6Mo_7O_{24}.4H_2O$ and 62.5 g/L Na_4EDTA dissolved in sterile water in the listed order, heated to boiling, cooled to 60°C, adjusted to pH 6.5-6.8 with 5M KOH and autoclaved). To prepare *Aspergillus nidulans* minimal medium agar plates, 50 mL 20x salts, 1 mL trace elements and 10 g glucose were added to 800 mL of water and adjusted to pH 6.5 with 5M KOH, before being adjusted to a 1 L volume. 10 g Agar No. 1 was added to this and the solution was autoclaved to produce solid media. During pouring, plates were amended with timentin and hygromycin, both to a final concentration of 100 µg/ml. *Aspergillus nidulans* minimal medium agar plates were produced fresh when needed and were used on the same day.

Once set, 100 µl dH_2O was pipetted onto each agar plate and membranes from the previous IM plates were transferred to them using sterile tweezers. These plates were left unsealed and incubated at room temperature for 10-14 days.

The *Z. tritici* transformation protocol was generally highly successful, requiring only one attempt to produce most of the desired fluorescent strains. However, early concerns regarding the effectiveness of the process (due largely to unexpected drops in the OD_{600} readings during the monitoring of initial Agrobacterium suspensions) did lead this process being repeated for several of the tested fluorescent *Z. tritici* strains. No clear differences were observed between fluorescent strains produced from the first and second of these transformations.

Selection of transformants

Z. tritici colonies that developed on membranes over selective plates were selected and transferred to YPD plates amended with timentin and hygromycin, both to a final concentration of 100 µg/ml. These colonies were allowed to grow at 17°C for 4-6 days before undergoing cPCR testing. This testing used DreamTaq (ThermoFisher) according to the manufacturer's instructions and the forward and reverse primers for the *GFP* and *mCh* coding sequences (as previous cPCR reactions had been challenging due to the length of the entire construct region).

All colonies were then examined against a YPD background using a Leica M205 FA Stereomicroscope (Leica, Milton Keynes, UK) running Leica Application Suite (LAS) X software, with standard eGFP and mCherry filters. The most highly fluorescent colonies were noted and their images were saved for later reference.

Transformants that showed fluorescence were harvested into 50% w/v glycerol and stored at -80°C. These suspensions were then examined under a Leica FALCON 8 confocal microscope (Leica, Milton Keynes, UK) (van den Broek and Jalink, 2019) running LASX software, using the standard settings and filters for eGFP and mCherry. This allowed the visualisation of fluorescence in individual cells to ensure that fluorescence was consistent throughout the strain produced, and to more precisely compare levels of fluorescence between strains. Settings were re-optimised for the visualisation of each transformant.

Testing of selected transformants' virulence

The virulence of positive transformants was tested against the susceptible wheat variety KWS Cashel. At least four KWS Cashel plants (containing no known *Stb* genes) per transformant to be tested were grown for approximately three weeks (adapted for variable growth rates where necessary) in a 16-hour day, 8-hour night cycle at a temperature of 22°C and ambient humidity. *Z. tritici* strains used in inoculations were cultured on YPD agar plates and grown for 4-7 days at 17ºC.

Fungal blastospores were harvested using sterile loops into 5 mL of 0.1% Silwet L-77 surfactant (Momentive Performance Materials, Waterford, NY, USA) in H_2O, then diluted to a concentration of 10^7 spores per mL using measurements from a haemocytometer. Spores were inoculated onto the third leaf of each plant using cotton buds, ensuring an even layer of moisture on the leaf surface with four passes per leaf.

Post-inoculation plants were transferred to 17°C and the same 16-hour day, 8-hour night cycle. Non-inoculated leaves were trimmed to ensure light access to inoculated leaves. After inoculation, plants were placed in high humidity boxes for three days to allow fungal development (in some cases this period had to be extended due to the lack of alternative space available to transfer plants to). Growth and monitoring of the infection development on these plants then proceeded for 28 days post inoculation (dpi).

It should be noted that although the inoculation procedure carried out using these transformed isolates was very similar or identical to those described in previous chapters, a shortage of available growing space during these experiments required the use of suboptimal growth and

inoculation facilities. This may have negatively affected the results obtained from this work.

Phenotyping of inoculated plants

Visual assessment of symptoms of necrosis, chlorosis and pycnidia development began ten days after seedling inoculation by *Z. tritici* for each plant. Leaf status was recorded as no infection (i.e. clean), chlorosis present (showing yellow chlorotic tissue but which had not yet progressed to necrosis), necrosis present (where necrotic lesions were visible), chlorosis with pycnidia (chlorotic symptoms present with small black pycnidia visible on the inoculated leaf surface) or necrosis with pycnidia. The first date on which chlorosis or necrosis was seen was used to determine the "days until symptom development" symptom value, while the date on which pycnidia were first noted was used to determine the "days until pycnidia development" symptom value. Assessments were carried out three times a week at regular intervals until 28 dpi.

At 28 dpi the "percentage leaf area covered by symptoms" and "percentage leaf area covered by pycnidia" were recorded. The values for each leaf were rounded to 0, 20, 40, 60, 80 or 100%. Photographs were taken in case needed for later verification of results. Results were analysed and verified by single-factor ANOVA tests using the R (R Core Team, 2017) and Excel Data Analysis tools.

Observing stomatal closure and fungal penetration through stomata

Plants to be used for stomatal closure tests included KWS Cashel susceptible controls, NIL pairs produced by our collaborator Cyrille Saintenac (similar to those produced for Battache *et al.* (2022)), and resistant lines known to carry the *Stb* genes of interest. These plants were grown at 17°C in a 16-hour day, 8-hour night cycle throughout the experiment, including three weeks prior to inoculation.

A day before inoculation took place, leaves to be inoculated were affixed to aluminium inoculation tables using double sided sticky tape and rubber bands (which also defined the area to be inoculated and scored). Leaves not to be inoculated were trimmed to ensure light access to inoculated leaves.

Z. tritici isolates used in inoculations were cultured on antibiotic-free YPD agar plates and grown for one week at 16ºC. Fungal blastospores were then harvested using sterile loops into 5 mL of 0.1% Silwet L-77 surfactant in H_2O and diluted to a concentration of 10^7 spores per mL using haemocytometry measurements.

Leaves were then spray inoculated using small spray bottles to avoid damage to leaf surfaces for later imaging. Plants were left in high-

humidity clear plastic boxes for three days post inoculation to encourage spore germination.

Inoculated leaf sections were then harvested at 1-, 3-, 5-, 8- and 12-dpi, and stained with 0.1% Calcofluor White for 30 sec before being washed off in distilled water. These leaf sections were examined under a Leica FALCON 8 confocal microscope (Leica, Milton Keynes, UK) (van den Broek and Jalink, 2019) using LASX software. Images were taken over an area of 586 µm X 586 µm (a total of 200X magnification), consisting of Z-stacks of 5, 10 or 15 evenly spaced images depending on the variation in focal depths necessary to visualise all fluorescent *Z. tritici* on the leaf surface. Images were taken at 1024 X 1024 pixels produced from averaging a line read depth of 5 to reduce noise. Standard EGFP, mCherry and DAPI filters for this confocal system were used to visualise GFP, mCherry and Calcofluor White channels respectively. The eGFP filter used provides an excitation wavelength of 489 nm, while the mCherry filter uses an excitation wavelength of 587 nm. The DAPI filter used to visualise Calcofluor White provides an excitation wavelength of 405 nm. The detection wavelengths used were generally set to approximately 430-480 nm for the DAPI channel, 495-530 nm for the eGFP channel and 585-630 nm for the mCherry channel.

The total number of images produced for each leaf examined in this way varied dramatically between leaves. The number of images per Z-stack varied depending on the number of different focal depths required to produce clear images of all objects of interest on the leaf, and the number of channels varied depending on the expected fluorescence's of the *Z. tritici* used. As this was intended as a preliminary study, generally only one image was taken of each from a single leaf at each time point for each wheat genotype-*Z. tritici* strain interaction. This leaf location photographed for this image was selected at a position that showed good coverage of both spores (regardless of germination states) and stomata (regardless of opening). In a small number of cases where only one of these features could be framed at a time, additional Z-stacks were taken to ensure that records of both elements of the leaf surface were recorded (although the images with the optimal visualisation of pycnidia were used for analysis).

These images were then examined to identify fungal spores, fungal spore germination and stomatal penetration attempts. These examinations were performed by eye, with any spore that seemed to show evidence of branching or significant lengthening compared to initial spores being considered to have germinated, any that seem to reach and attempt to push into the central ostiole of any stomata considered to have made a penetration attempt, and any hyphae that made a penetration attempt for which visible Calcofluor White staining cut off at the stomatal opening without a visible end to their hyphae

were considered to have made a successful penetration attempt. Ideally this would have been determined through the presence of fluorescent fungal matter in the sub-stomatal cavity, but suboptimal fluorescence results in this study made such assessments extremely difficult.

Results

Fluorescent *Z. tritici* strains were successfully produced

Isolates RResHT-1, -22, -42, -44, -45, -52, and IPO 323 were transformed with the GFP and/or mCherry expression constructs and examined through cPCRs and microscopy. Initial cPCR testing of *Z. tritici* transformant colonies was unsuccessful, with only six of the total 38 tested mCh transformed colonies producing recognisable bands, and all GFP colonies failing to produce bands at all. As many of these colonies were shown to be fluorescent, this is assumed to be due to issues with the PCR reaction using this set of primers, as it seems that the correct fluorescence genes must have been present to produce this fluorescence. As the cPCR procedures used here were originally designed for *E. coli* and thus used whole cells in the PCR reaction, it is possible that the fungal cell wall proved a major impediment to this procedure. Breaking down this wall (e.g. using high temperatures for an extended period) or fully purifying fungal DNA before the cPCR reaction may have made this process more successful, but was not tested here as the colony fluorescence could be easily assessed by other means.

All examined colonies showed the correct fluorescence for GFP and mCherry at the colony level when examined under a stereomicroscope (expected fluorescence for Calcofluor White is a 475 nm emission wavelength from a 380 nm excitation wavelength, for GFP is a 509 nm emission wavelength from a 395 nm excitation wavelength, and for mCherry is a 610 nm emission wavelength from a 587 nm excitation wavelength). Each individual transformation produced many transformed *Z. tritici* colonies (several dozen to several hundred after extended incubation to allow maximum colony development). Subsets of these colonies were tested for fluorescence as described, including 4-16 colonies for each fluorescent strain (with higher total numbers for strains produced from the repeated initial and second transformations, particularly strains derived from the isolates RResHT-1, -44 and -52).

Unfortunately, levels of fluorescence could be significantly affected by differential levels of melanisation and growth between *Z. tritici* isolates. Therefore, additional measurements were made using confocal microscopy to examine individual *Z. tritici* spores. In all tested cases, good visible fluorescence responses were noted from the GFP or

mCherry expressed in all cells examined. Figure 1 shows examples of such fluorescence.

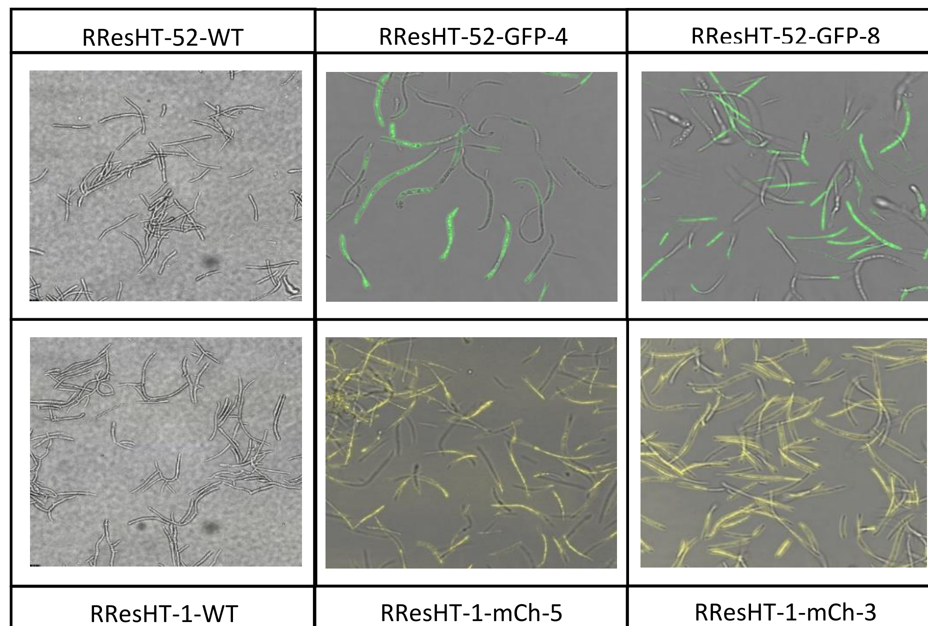

Figure 1: Examples of confocal fluorescence images collected of *Z. tritici* blastospores in vitro using the EGFP and mCherry filters. The fluorescence of both RResHT-1 mCherry and RResHT-52 GFP strains can clearly be seen. High resolution images of the parental wild type isolates were taken as negative controls, each using both GFP and mCherry filters along with Bright Field images that provided most of the visual data for the WT images.

Testing of fluorescent strains on susceptible KWS Cashel wheat suggests that virulence is retained in fluorescent *Z. tritici*

The most highly fluorescing *Z. tritici* strains identified through confocal microscopy were tested on the susceptible wheat variety KWS Cashel (containing no known *Stb* genes). The results for these tests are given in Table 2, along with statistical comparisons between the results from mock and fluorescent strain inoculations. For all four measured phenotypes for disease symptoms, every fluorescent strain developed caused higher average levels of virulence-related symptoms than were present in mock inoculated or non-inoculated plants (i.e. less time to symptom and pycnidia development post inoculation, and greater coverage of inoculated leaves with chlorosis, necrosis and pycnidia after 28 days). As this data was not normally distributed (due to large numbers of plants showing low levels of infection symptoms compared to the averages for each phenotype measured), non-parametric Kruskal-Wallis tests were conducted using R. For the collective tested lines of RResHT-1-mCh-1, -3 and -4, RResHT-52-GFP-3 and RResHT-52-mCh-1 these tests gave p = 0.00016 for days post inoculation to

symptom development, $p = 0.031$ for days post inoculation to pycnidia development, $p = 5X10^{-6}$ for % leaf symptom coverage 28 days post inoculation and $p = 6X10^{-6}$ for % leaf pycnidia coverage 28 days post inoculation. These highly statistically significant results support the clear variance in symptom levels seen in this data set, indicating that the transgenic strains used generate significantly different levels of disease symptoms. Individual comparisons of each strain to mock inoculations were made using non-parametric Kruskal-Wallis tests (as most datasets included too many low values to be normally distributed, giving Shapiro-Wilk test results of $p < 0.05$ for all phenotypes measured). These results (see Table 1) revealed that all strains show significantly higher symptom coverage at 28 dpi than mock inoculated plants, and all except RResHT-1-mCh-3 and -4 show similar significance for the pycnidia coverage at this time point. This lack of statistical significance for these strains is likely due to the low levels of pycnidia generally produced in these tests and the relatively low numbers of leaves tested for these lines, as the large differences in average seen to the mock result suggest that these strains do possess functional virulence. The small amount of pycnidia coverage seen on one mock leaf is assumed to be due to contamination of the mock inoculated leaves, either during the inoculation of neighbouring leaves or due to splashing during watering, caused by the high leaf density in this experiment. In either case, it is recommended that barriers are introduced between leaves inoculated with different isolates are used in future testing to prevent such contamination. Statistical analysis could not be completed for the days to pycnidia development due to the fact that many leaves did not develop pycnidia by 28 dpi, meaning that sample sizes were too small.

These tests are sufficient to suggest that some level of virulence was retained in transformed *Z. tritici* strains. However, this data does not demonstrate that the aggression of these *Z. tritici* isolates are unchanged from the wild type isolates. Confirming this would require an additional set of tests using plants of relevant wheat lines inoculated with fluorescent strains and the wild type isolates used to produce them. While such experiments were not conducted due to time and space restraints in this preliminary work, conducting such tests should be a priority for future work.

Leaf surface interaction analysis using confocal microscopy

In order to investigate the mechanisms of *Stb5*-mediated resistance, Chinese Spring NILs CS- (no known *Stb* resistance genes) and CS+ (containing *Stb5*) were tested alongside susceptible KWS Cashel and *Stb5*-containing Synthetic 6X control lines. Tests were conducted using fluorescent strains derived from the isolate RResHT-1, which is known

to be virulent against KWS Cashel but avirulent against Synthetic 6X, and RResHT-52, which is known to be virulent against both lines (Tidd *et al.*, 2023).

Stomata opening and fungal hyphae development on the inoculated leaves were examined using confocal microscopy. Visualisation in some cases was made more difficult by background fluorescence, especially for transformed strains expressing the mCherry fluorophore. The lower band of chlorophyll autofluorescence emission wavelengths (approx. 648 nm) overlaps with the upper band of mCherry fluorescence emission wavelengths (approx. 650 nm), which should theoretically result in minimal overlap. However, in this experiment significant background fluorescence was observed, suggesting that other compounds present in wheat leaves may have produced further autofluorescence overlapping with the mCherry range. Appropriate Z-stacks of 5, 10, 15 or 20 images (dependent on the total distance covered by the Z-stack) were collected for a range of wheat line – *Z. tritici* strain comparisons. As this preliminary experiment represents only one leaf per wheat line-fluorescent *Z. tritici* strain interaction per time-point, further statistical analysis has not been included for these results.

Stomatal opening appears more affected by environmental variation than fungal inoculation

In mock inoculated plants, the proportion of open stomata identified generally increased from the 3 dpi to 8 dpi, before being greatly reduced again on 12 dpi. The open or closed state of stomata was measured by eye – stomata that showed any gap between the guard cell walls on all focus depths were considered “open”, as low numbers of Z-stack planes and high fluorescence/Calcofluor White levels in some images made the differentiation of partially open stomata difficult.

In general, the proportion of stomata identified as being open was slightly higher in leaves inoculated with the wild type *Z. tritici* isolates RResHT-1 and RResHT-52 than the fluorescent RResHT-1-mCherry and RResHT-52-GFP strains derived from them. Differences between stomatal opening in CS+ and CS- NILs were minimal overall, although some trends are suggested by these results. The proportion of open stomata in KWS Cashel was generally higher than average when RResHT-1 derived isolates were used, whereas other wheat genotypes showed greater variability. When RResHT-52 derived strains were used, day one post inoculation opening levels were very low (zero for all genotypes other than CS+), and CS- showed consistently moderate to high opening levels from three days post inoculation, whereas variability in other genotypes was higher. Initial low stomatal opening levels were also seen for co-inoculations with RResHT-1-mCherry and

Table 2: The *p*–values derived from Kruskal-Wallis tests comparing mock inoculations to each transgenic *Z. tritici* strain, with the average symptom development caused by tested strains compared to mock inoculations carried out in this study, and to data from wild type isolates on KWS Cashel (Tidd *et al.*, 2023). Grey shading indicates insufficient sample sizes to carry out statistical tests. The small amount of pycnidia coverage seen on one mock leaf is assumed to be due to accidental contamination during inoculation or watering.

Z. tritici strain with which KWS Cashel plants were inoculated		Days to initial symptom development	Days to initial pycnidia development	Final leaf surface % symptom coverage	Final leaf surface % pycnidia coverage
Mock inoculation	Average symptom measurement	17	28	16	1
RResHT-1-mCh-1	Average symptom measurement	13	22	53	27
	P-value from comparison to mock	0.057	-	0.048	0.001
RResHT-1-mCh-3	Average symptom measurement	13	18	80	20
	P-value from comparison to mock	0.038	-	0.018	0.053
RResHT-1-mCh-4	Average symptom measurement	13	19	65	15
	P-value from comparison to mock	0.101	-	0.018	0.053
RResHT-52-GFP-3	Average symptom measurement	13	20	100	40
	P-value from comparison to mock	0.038	-	0.018	0.005
RResHT-52-mCh-1	Average symptom measurement	11	18	64	48
	P-value from comparison to mock	0.001	-	0.002	0.000
RResHT-1-WT	Average symptom measurement	15	18	80	45
RResHT-52-WT	Average symptom measurement	13	18	100	0

RResHT-52-GFP, although with a very rapid rise in opening levels by 3 dpi afterwards. Overall, the high variability in these results may also suggest that environmental factors present in the plant's growth conditions (e.g. related to watering periods) influenced stomatal opening levels. Supplementary Table 1 shows the percentages of stomata that were scored as open on each leaf.

Spore germination rates do not appear to be influenced by the presence/absence of *Stb5*

Mock inoculated plants generally did not show any spores, as expected, with the exceptions of some possible imaging artefacts and some spores that appeared to be visible on mock inoculated Synthetic 6X leaves. This is likely due to unintentional contamination in the spray inoculation process.

For leaves inoculated with *Z. tritici* spores, there was a clear trend for spore germination rates to start out low at 1- and 3-dpi, then increase to their maximum levels around 8-dpi. Successful germination rates in RResHT-52 derived *Z. tritici* strain were generally higher than in RResHT-1 derived strains, as expected for the more virulent strain. However, no single strain showed high variation in germination rates between the Chinese Spring NIL pairs. *Stb5* containing plants inoculated with the mixture of *Z. tritici* strains showed lower proportions of spore germination than those with no *Stb* resistance genes, but this difference has effectively ended by 3-dpi and the remaining results showed no such differentiation. This trend was not seen in the results from single strain inoculations and is not expected to be seen in further repeats of this experiment as little evidence exists to suggest the prevention of hyphal germination on leaf surfaces as a likely mechanism for *Stb* resistance genes. Supplementary Table 1 shows the percentages of spores present on each wheat leaf that have germinated. Figure 2 shows late stage germinated spores from a CS+ (*Stb5*) leaf.

In general, low sample sizes of spores on leaves in these inoculation experiments made these results less reliable and more difficult to interpret then we would prefer, especially for mixed infections. This is likely due to the spray inoculation method used here to avoid causing damage to the leaves. In future experiments higher spore concentrations should be used to address this issue.

Percentage of stomata reached by epiphytic hyphae may be higher for *Stb5*-containing wheat lines inoculated with the virulent *Z. tritici* isolate RResHT-52 and its derived GFP-expressing strains

Fungal structures generally not observed from mock inoculations, except for one case in Synthetic 6X, which is assumed to be due to accidental contamination during inoculation. For both RResHT-1 derived strains, higher percentages of stomata were reached by at least one growing hyphae in fluorescent strains than wild type (WT) isolates, although this was reversed for RResHT-52 derived strains. All results show significant variability and few clear consistent trends. The proportion of stomata that were reached by epiphytic hyphae appeared to be higher in CS+ (*Stb5*), particularly for RResHT-52-GFP, with levels also being unexpectedly high for the *Stb5* containing line Synthetic 6X, but the relatively low numbers involved make it difficult to be certain that this trend is statistically significant. If real, this trend is surprising, as although RResHT-52 was expected to be virulent on *Stb5* containing lines it was not expected to be less virulent on other lines. This could suggest that *Stb5* responses are stimulated by RResHT-52 to enhance symptoms or virulence. Such strategies have been identified for necrotrophs in other systems, although usually in connection with the hypersensitive response to cause necrosis rather than to locate stomata or secure entry into the leaf (Lorang, 2018).

The numbers of stomata that were reached by epiphytic hyphae were also relatively low in co-inoculation experiments, possibly due to overall lower spore counts. Supplementary Table 1 shows the percentage of stomata that were reached by epiphytic hyphae at each time point.

Stomatal penetration attempts may be delayed on *Stb5*+ wheat lines inoculated with the virulent isolate RResHT-52, and in wheat plants inoculated with avirulent RResHT-1

There were no penetration attempts from RResHT-1 derived strains until 12-dpi, although the proportion of penetration attempts at later time points becomes generally high. This delay is not unexpected as *Z. tritici* RResHT-1 was expected to be the less virulent isolate on *Stb5* containing wheat lines, but the relatively low number of stomatal penetration attempts on all wheat lines by all *Z. tritici* strains and isolates does make it difficult to directly compare results on different wheat lines.

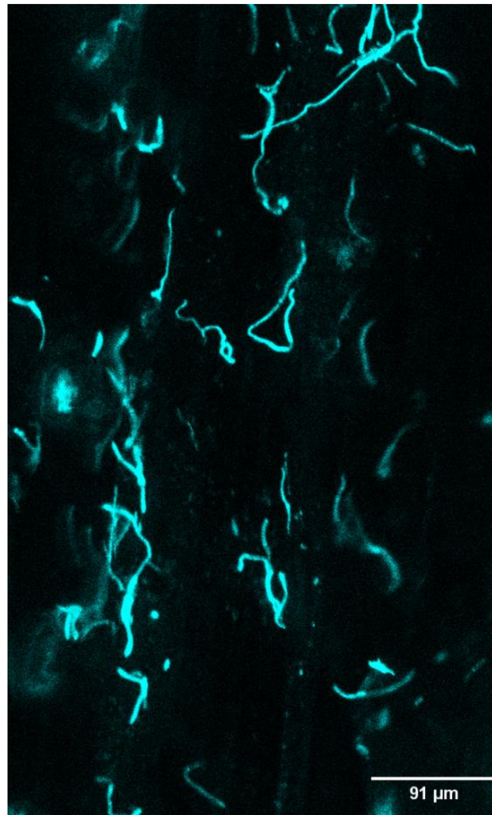

Figure 2: Late stage RResHT-52 spores at 30 dpi on CS+ (*Stb5*) leaf surface, visualised using Calcofluor White stain and the DAPI confocal filter, demonstrating advanced *Z. tritici* spore germination.

For RResHT-52 derived strains, penetration attempts began earlier at 5-dpi, but appear to have been delayed in CS+ (*Stb5*) NIL line plants compared to CS- (no *stb5* present) NIL line plants, and penetration attempts observed in CS+ plants only after 12-dpi. The mixed inoculations showed no stomatal penetration attempts, possibly because of the low spore counts in the mixtures.

Unfortunately the majority of leaves from all wheat lines and inoculation sets did not show stomatal penetration attempts – the resulting low sample size for this data is the most likely explanation of counterintuitive results, such as the comparatively high ratios of penetration attempts for Synthetic 6X inoculated with RResHT-52-GFP and the lack of penetration attempts resulting from co-inoculations. Future repeats of this experiment using the same tools conducting confocal measurements at later time-points post inoculation and using larger numbers of experimental plants in staggered screens are likely to clarify these results and allow more useful analysis. Supplementary Table 1 shows the percentage of stomata in contact with epiphytic hyphae that have undergone penetration attempts. Figure 3 shows two stomata undergoing fungal penetration attempts on a leaf surface.

Low frequencies of successful stomatal penetration attempts were observed in all wheat lines

When inoculated with RResHT-1 derived strains, no leaf showed successful penetration attempts until 12-dpi. No successful penetration occurred in the CS+ line while one event did occur in the CS- line, but one also occurred in the Synthetic 6X line, which also carries *Stb5*.

For plants inoculated with RResHT-52 derived strains, the first successful stomatal penetrations began to occur at 5-dpi. Although the numbers of successful penetration attempts from RResHT-52 derived strains were very low, one successful penetration still occurred in the CS- NIL line. The CS+ NIL line experienced no successful stomatal penetrations with strains derived from either *Z. tritici* isolate used here.

The wheat line Synthetic 6X also experienced successful penetrations, despite carrying *Stb5*. Inoculations using mixed RResHT-1-mCh and RResHT-52-GFP strains did not produce any successful penetration attempts by 12-dpi. Supplementary Table 1 shows the proportion of stomatal penetration attempts that were successful on each wheat line at each time point.

Discussion

Previous studies (Battache *et al.*, 2022) have demonstrated the value of fluorescent *Z. tritici* fungal strains to research aimed at analysing leaf surface interactions, due to the easily visualisable nature of the fungus. These studies have also suggested that stomatal closure is a likely resistance mechanism for several *Stb* genes, which could be further investigated using *Z. tritici* isolates with a greater variety of fluorescence profiles. This project has successfully produced several strains of fluorescent *Z. tritici* expressing GFP and mCherry fluorescent proteins. These fluorescent strains were derived from WT isolates either virulent or avirulent to Synthetic 6X, Synthetic M3, Kavkaz-K4500, Tadinia, Estanzuela Federal and Balance. These strains can be used as tools in further research, particularly research analysing infection patterns and resistance behaviour at a physiological level.

Unfortunately, due to limits in the time and resources available, it was not possible to fully test the purity or stability of the fluorescent *Z. tritici* strains produced here during this PhD. It would be an advisable first step for any future project using these resources to test these traits. Sequencing the strains produced here would optimally confirm that the fluorescence constructs had integrated correctly with the fungal genome, as well as confirming integrated copy numbers and ensuring that genes known to be important in *Z. tritici* had not been disturbed by this integration. Simpler methods, such as southern blotting or even a cPCR reaction on purified DNA, would likely provide cheaper methods

of verifying that the correct construct was present in fungal DNA if desired, although these methods would provide less information on the location of such inserts. Although an understanding of the genomic location of fluorescence inserts in the *Z. tritici* strains produced here would help to produce estimates of the likely stability of the transformation, it would also be desirable to re-plate the *Z. tritici* strains through several fungal generations and compare initial and final fluorescence levels to ensure that this transformation was fully stable.

In general, the mCherry expressing *Z. tritici* strains produced here were observed to have higher fluorescence than GFP expressing strains. This could suggest that mCherry is a more effective fluorophore for use in *Z. tritici*, possibly due to improved production or survival of the protein in the fungi's cells, or due to better penetration of this fluorescence wavelength through fungal cell walls. However, it is important to consider whether this will remain true when attempting to visualise *Z. tritici* infections on leaf surfaces.

Both chlorophyll (typically the strongest source of autofluorescence in leaves) and mCherry fluoresce in the red spectrum, although the emission peak of chlorophyll fluorescence is at 683 nm and that of mCherry is at 610 nm. However, significant autofluorescence from leaf cells was still seen within the expected mCherry spectra in the confocal microscopy work carried out here. The maximum wavelength used in visualising mCherry fluorescence, 630 nm, may overlap with the minimum expected emission wavelength of chlorophyll at approximately the same value. The potential for this issue to emerge has been noted in other papers, although selection of the correct filters is generally sufficient to prevent it (Lim *et al.*, 2019). However, the chlorophyll fluorescence at this level should have been very low. Some overlap may lead to visible background fluorescence if the levels of chlorophyll present were vastly higher than those of mCherry expressed in the *Z. tritici* transformants, but the fact that this background autofluorescence could not be easily removed through alteration of the detection parameters may suggest that other plant compounds also contributed. The total autofluorescence of plant leaves is known to cover most of the wavelengths utilised by common fluorophores, including the longer red wavelengths (Harter *et al.*, 2012), with components such as anthocyanins and alkaloids having fluorescence profiles that can overlap with that of mCherry (Müller *et al.*, 2013). It is possible that host resistance responses to *Z. tritici* infection could have led to the production of additional fluorescent compounds that could have concealed the mCherry fluorescence.

Although the reason for this background fluorescence could not be accurately determined in this study, it was clear that the GFP fluorescent *Z. tritici* strains developed here were more easily visualised

then the mCherry fluorescent strains, despite the average advantage in fluorescence intensity that mCherry strains showed *in vitro*.

Within a given set of *Z. tritici* isolate – fluorescent protein combination, fluorescence levels generally do not vary greatly against a blank microscope slide background, suggesting that as expected only one copy of the relevant fluorescent protein gene has been inserted into each genome. This fluorescence was expected to positively correlate with *Tub2* tubulin gene expression, which may be highest in growing hyphae and spreading infection due to the role of tubulin in nuclear division (Martin *et al.*, 1997; Rogers and Egan, 2023) and hyphal growth (Roberson and Vargas, 1994; Zhao *et al.*, 2014). However, this seems unlikely to have had a major effect as *Tub2* is generally considered to be constitutively expressed (Drummond and Cross, 2000; Schuster *et al.*, 2015).

The fluorescent *Z. tritici* lines produced in this project show that the methods used here to produce fluorescent protein expressing constructs and integrate them into the *Z. tritici* genome do not generally inhibit the ability of the pathogen to infect susceptible plants. This is likely a positive sign that the overall virulence of these isolates has not been affected by the expression of fluorophores, which will enable them to be used to examine a number of *Stb* resistance genes. This is as expected, due to the low likelihood of a randomly inserted genetic sequence such as those coding for GFP or mCherry being inserted into genes necessary for virulence in the genome of *Z. tritici*. Additionally, the *Z. tritici* genome is known for being highly flexible, with genome size changing significantly between isolates due to the retention or loss of at least 8 accessory chromosomes (Goodwin *et al.*, 2011). This level of genetic flexibility may suggest that *Z. tritici* is at least partially pre-adapted to survive the insertion of new genes with minimal loss of functionality, especially if changes could be targeted to such accessory chromosomes.

The success of the lines produced here shows that *Z. tritici* can adapt relatively well to the addition of multiple types of fluorescence protein encoding genes to its genome. This is unsurprising given the genetic flexibility of *Z. tritici* (Goodwin *et al.*, 2011; Berraies *et al.*, 2013; Mekonnen *et al.*, 2020) and the success of previous experiments using GFP-transformed *Z. tritici* (Battache *et al.*, 2022). However, it suggests that this set of fluorescent strains can be expanded with further fluorophores. Additionally, as all tested *Z. tritici* strains led to the successful production of transformants through the relatively simple transformation methods described here, it seems likely that this collection could be easily expanded with additional transformed isolates using the Gibson Assembly constructs already produced.

This flexibility with the ability to insert marker genes will be useful, as the isolates currently used in this research all originated in the UK whilst those used in previous research (Battache *et al.*, 2022) were produced using established IPO isolates (except for CFZ008, collected in France in 2016), which generally do not have virulence or fungicide resistances equal to modern wild type isolates. Adding modern strains from other geographic areas and checking for similarities and differences in the common methods of infection and the resistances which operate against them would be highly desirable. Overall, the methods described here suggest that future research in this field could yield a deeper understanding of this pathosystem which, despite significant study, has so far been lacking.

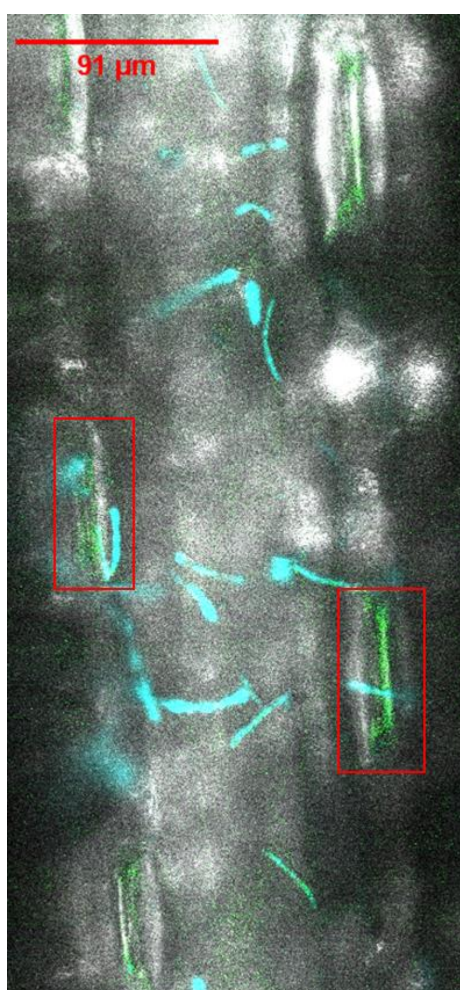

Figure 3: The *Z. tritici* strain RResHT-52-GFP infecting Synthetic 6X wheat leaves at 12 dpi. Two highlighted stomata in this image show signs of penetration attempts. This image was compiled from images taken using DAPI and EGFP filters to produce the coloured sections (stained with calcofluor white and GFP, respectively). Bright field images were also used to visualise the background. The brightness, contrast and false colour of the channels compiled into this image have been modified using ImageJ software for clarity.

Preliminary experiments using the fluorescent *Z. tritici* strains produced here were also carried out as part of this project. These experiments included testing fluorescent *Z. tritici* strains derived from RResHT-1 (avirulent against *Stb5*) and RResHT-52 (virulent against *Stb5*) transformed with mCherry and GFP, respectively. Multiple symptoms were then measured at 1, 3, 5, 8 and 12 days post inoculation. These symptoms included rates of stomatal opening, spore germination, stomata reached by epiphytic hyphae, stomatal penetration attempts made, successful stomatal penetration attempts, secondary sub-stomatal cavity colonization, early stage pycnidia and development into young and mature pycnidia. Unfortunately symptoms beyond successful stomatal penetration were not visible using these fluorescent *Z. tritici* lines by day 12.

It was hoped that the examination of these infected leaves would reveal significant differences between the CS+ and CS- NILs (Chinese Spring NILs containing and lacking the *Stb5* gene, respectively). Such differences could have been interpreted to determine some of the resistance mechanisms utilised by this resistance (e.g. stomatal closure, as in other *Stb* resistance genes (Battache *et al.*, 2022).

Unfortunately, differentiation between these NILs was generally poor in this preliminary study, while variation within each line was high for many of the measured responses. This makes it very difficult to draw firm conclusions from these preliminary experiments. Further experiments using these fluorescent strains should be modified accordingly to provide clearer data by solving the problems that were encountered during these preliminary tests.

Limitations of this study and future methodology improvements

The first issue that became clear during these trials was the low spore count present on many of the inoculated leaves even at 1- and 3- dpi. This is likely due to the spray inoculation method chosen for these inoculations in order to minimise damage to the leaf surfaces that were intended to be used in later confocal microscopy. In these preliminary tests, 10^7 spores per ml were used (as in Tidd *et al.*, 2023) to enable comparisons of infection symptoms between susceptible and *Stb5*-containing resistant wheat lines infected *Z. tritici* isolates RResHT-1 and RResHT-52 or fluorescent strains derived from these isolates in Tidd et al. (2023) and this work, respectively. However, although adequate for cotton bud inoculation, the 10^7 spores per ml concentration is clearly not suitable for spray inoculations, possibly due to less complete leaf coverage from spray inoculations or spores being trapped in the spray mechanism. Therefore, higher spore concentrations should be used in future experiments. Alternatively,

different sprayer models could be trailed to identify those that function more effectively using this spore concentration.

The second major issue present with this preliminary research was that many of the more advanced symptoms of infection for which our images were analysed simply did not occur by 12-dpi using our *Z. tritici* isolates. This likely indicates that either more aggressive *Z. tritici* isolates are needed, or that a longer infection time course would be more suitable for this experiment (which is likely to be a more practical solution when working with broadly effective *Stb* resistance genes such as *Stb5*). It was noted that by 12-dpi, only mild chlorosis was visible at the macroscopic level on some of the susceptible KWS Cashel leaves. However, by 30-dpi, all leaves except for the mock inoculated Synthetic 6X leaves possessed very high levels of necrosis with relatively low levels of pycnidia development (which was mostly confined to leaves inoculated with RResHT-1 derived isolates). Furthermore, the lack of fluorescence in both fluorescent *Z. tritici* isolates by this stage likely indicates that they were no longer growing. This would suggest that inoculated leaves had died between 12- and 30-dpi, possibly due to a combination of the presence of *Z. tritici* and environmental factors, making it difficult to measure many of the symptoms of interest in the final set of screens. Therefore, it would be advisable to introduce additional checks to future screens between approximately 16- and 21-dpi to ensure that advanced symptoms can be measured before leaf death occurs. Modifying the watering schedule or identifying ways to improve the relative humidity and temperature control of the experimental conditions may also reduce rates of leaf necrosis to enable fungal infections to reach late stage symptoms before full necrosis occurs.

The third issue with these preliminary results is the variability of the proportion of open stomata in mock inoculated plants (especially in the final 12-dpi measurements), which suggests that environmental effects outside of the *Z. tritici* inoculations influenced stomatal opening in ways that likely acted as a confounding factor for these results. The most likely explanation for this is that the facilities used for this experiment struggled to maintain the proper levels of humidity, as the plants used in these tests were noted to require more watering than previous wheat seedlings, including those of the same lines, grown in other facilities by the author. If this did lead to greater levels of stomatal closure than would be caused by the host plants defence reaction to the *Z. tritici* infection, this could have contributed to the poorer than expected results obtained from other measured infection and infection response indicators. It is possible that this issue could be fixed with more frequent (daily) watering, or by conducting future runs of this experiment in an alternative facility with higher relative humidity. It may also be advisable to adjust plant growth period and harvesting times to

enable watering to take place several hours in advance of leaf harvesting, to ensure that stomata have time to open in response to water availability. It is also possible that the arrangement of leaves to be inoculated on aluminium tables could have influenced stomatal opening by reducing gas exchange through stomata on the underside of the inoculated leaves, which were resting on the inoculation tables. However, such effects were expected to be minor due to the reduced number of stomata typically present on the underside of cereal leaves compared to the upper surface. It may still be advisable to ensure this is not an issue before larger screens are designed by comparing stomatal opening results from plants maintained in this setup to results gathered from the leaves of free-standing plants that have been similarly inoculated.

The fourth issue encountered with the preliminary experiment was the difficulty of the microscopy itself. Although some of this difficulty was likely unavoidable (caused by the contours of the leaf surfaces themselves), the issue was exacerbated by the lower than expected levels of fluorescence in the transgenic *Z. tritici* strains compared to the background autofluorescence of the wheat leaves. This was particularly problematic for isolates carrying the mCherry fluorophore, which has substantial overlap in its fluorescence spectra with observed leaf autofluorescence and thus can be difficult to differentiate from the background fluorescence. This issue is mitigated by the use of calcofluor white to highlight spores using externally binding fluorophores with alternative spectra, but can still make differentiating the alternative labelled spores difficult in mixed infections. It is possible that in future measurements, background chlorophyll fluorescence could be measured and used as a control, with the average level of background fluorescence being removed from all sections of the image. This has allowed mCherry to be used as an effective marker for chloroplast protein production in Kim *et al.*, 2020. However, performing this compensation correctly at each focal depth for each in-focus leaf section would likely require a significant increase in the level of processing and analysis required for these images. Alternatively, further fluorescent isolates could be produced using alternative fluorescent proteins that do not overlap with the fluorescent spectra of wheat leaves, or GFP-transformed isolates could be used for single isolate studies.

In addition to these issues, limitations in the time and resources available for this project placed limitations on the extent to which the fluorescent strains produced could be examined and verified. For example, it would have been beneficial to confirm the stability of the fluorescent strains produced over several fungal generations, particularly since *Z. tritici* contains several accessory chromosomes that can be lost, together with any insertions made into these

chromosomes. The strains produced here will be of much less use if they do not remain fluorescent through several replications.

It would also have been beneficial for the strains produced to be sequenced. Ideally sequencing would take place over their whole genomes, but benefits could also be derived from smaller scale sequencing of the areas around each fluorescence gene insertion using known primers for the fluorophores, promoters or terminators. The primary purpose of such data would be to determine whether the fluorophores had integrated into existing genes, particularly any that may be relevant to the pathogenicity of the fungus, which could cause alterations in the virulence profiles of these strains compared to the wild type isolates from which they were produced. However, data confirming the number and genomic location of inserts in each strain produced in this work may also have aided in the early identification of strains that were likely to display higher than average levels of fluorescence. This kind of data could also help to target future insertions using more sophisticated methodologies (e.g. CRISPR/Cas9 and homology directed repair (Begemann *et al.*, 2017)) to ensure maximum expression of the inserted genes.

It was also not possible within this work to fully test the aggressiveness or virulence profiles of the fluorescent *Z. tritici* strains produced. Although it seems unlikely that fluorescence will have any direct impact on the pathogenicity of the isolates used to produce these strains, both the resource drain of producing the additional fluorescent proteins and the possibility that pathogenesis-related genes have been damaged by the insertion of the fluorescence genes could have affected the resultant strains ability to infect wheat plants. It would therefore be desirable to test these strains against wild-type controls of the isolates from which these strains were produced on both known resistant and susceptible varieties of wheat, to ensure that the virulence profiles had not been altered. Broader testing against an array of wheat lines with varying levels of resistance would provide additional verification that the general aggressiveness of each strain towards other wheat lines had not changed in ways that may not be obvious in a strict examination of the impact of the fluorophores on fully resistant or susceptible lines.

In addition to sequencing these strains and testing their stability and aggressiveness, it would also have been useful to include more plants in this study, and to conduct several additional repeats. In addition to enabling the implementation of suggestions made throughout this chapter in later repeats, this would also have provided a stronger statistical basis for the assessment of the results obtained. This would have improved the likelihood of enhancing our understanding of the *Stb5* gene through this experiment.

With sufficient time and growing space available, this experiment could have also further enhanced our understanding of the mechanisms underlying *Stb* resistance genes in general and *Stb5* in particular through an examination of the ways in which plant growth conditions affects stomatal closure and any STB resistance provided by such a closure response. For example, growing plants in the dark for an extended period before and after inoculation or under water stress conditions could stimulate increased levels of stomatal closure, whereas compounds such as fusicoccin can directly trigger stomatal opening. Determining whether *Stb5* mediated stomatal closure responses were enhanced by or capable of overriding such stimulation could provide us with additional context relating to the mechanism through which *Stb5* operates and the circumstances under which this resistance should be optimally effective in the field.

In addition to this, it would be interesting to generate comparative data through the inoculation of *Stb5* resistance containing plants leaves that have undergone a wounding process, to enable hyphal entry into the leaves without the need to pass through stomata. This would allow an assessment of the relative importance of leaf surface defence mechanisms (including stomatal closure) against any internal defence mechanisms that may also be active in these lines, potentially providing additional information about this pathosystem. In the absence of internal defence mechanisms, an increase in disease symptoms among wounded plants could also be used as further evidence of the importance of stomatal closure in STB protection in wheat.

Similar experiments to those conducted and suggested here have already demonstrated their ability to provide useful data for several other *Stb* resistances, with the most significant success arguably being the investigation of the *Stb16q* mode of action by (Battache *et al.*, 2022). In this experiment, GFP-fluorescent *Z. tritici* strains were inoculated onto the leaves of wheat NILs containing or lacking the resistance gene *Stb16q*. Confocal microscopy was then used to monitor the interaction between leaf surface stomata and the fungal pathogen. This research revealed the highly significant role of stomatal closure in blocking *Z. tritici* infections during the early stages of infection. The increased growth of *Z. tritici* isolates infiltrated directly into leaves of plants expressing *Stb6* and *Stb9,* along with the increased levels of infection symptoms caused by the infiltrated isolates compared to those applied through more traditional inoculation methods, suggests that these resistances may work through similar leaf surface level mechanisms.

The fact that three tested *Stb* resistance genes seem to have mechanisms that operate at the leaf surface level suggests a high likelihood of similar mechanisms existing for at least some other *Stb* resistance genes. The fluorescent *Z. tritici* strains produced here can

be used for confocal microscopy examinations of non-compatible infection attempts of wheat lines containing other *Stb* resistance genes, to identify resistances that also operate through stomatal closure-based mechanisms. In addition to this, these strains express GFP and mCherry fluorophores, with the isolates used to produce these strains matched so that for each *Stb* resistance gene of interest, virulent and avirulent isolates are available with alternate fluorescence profiles. This will allow virulent and avirulent isolates to be differentiated in co-inoculations, enabling deeper investigations of resistance recognition mechanisms and the nature of the virulence genes fungi use to avoid them (e.g. active suppression vs passive avoidance of recognition). Internal plant resistance mechanisms can also be examined using fluorescent *Z. tritici,* e.g. to determine how far the infection is able to progress before being halted. This information could help to guide qPCR gene expression studies intended to determine the genetic composition of resistances by identifying genes upregulated around key sites for resistance responses. Where such genes are identified, the fluorescent strains produced here may also be useful for visualising the results of knock-out and complementation studies verifying their identities.

It may also be of interest to future researchers to compare the performance of the GFP expressing strains produced here to those produced and used in Battache *et al.* (2022). This would help to more accurately evaluate the potential of the GFP strains produced here for effective use in this type of research, as well as enabling the direct testing of *Stb5* against a broader diversity of fluorescent *Z. tritici* strains.

In addition to the fluorescent strains produced here, the sexual reproductive cycle of *Z. tritici* will make it possible for new isolates to be produced through mating fluorescent *Z. tritici* strains with wild type strains. Although this is unlikely to be as efficient as simply repeating the transformation process with a suitable new isolate when a specific virulence profile is needed, such a strategy may be useful for producing large numbers of new fluorescent isolates through fungal breeding programs, for example in order to test the broad heritability of virulence phenotypes. As virulent and avirulent isolates against a variety of known *Stb* resistance genes are available, these populations could make a good basis for performing future knockout, knockdown and complementation studies within the *Z. tritici* genome to examine the genetic basis for virulence. The fluorescence of populations produced from these strains would make the assessment of their interactions with leaf surfaces easier.

Further research into the host and pathogen genetics involved in this pathosystem will aid in maximising farming output across Europe by enabling the breeding of new wheat lines that are optimally resistant

against a wider array of *Z. tritici* isolates, and potentially by leading to new treatments for this pathogen. For example, recent data suggests that the asexual reproduction phase of the pathogen lifecycle may involve conserved RNAi machinery (Habig, Schotanus, *et al.*, 2021) – therefore although the lack of efficient dsRNA uptake by *Z. tritici* (Kettles *et al.*, 2019) renders direct RNAi and Host-Induced Gene Silencing (HIGS) ineffective for suppressing *Z. tritici* growth, it is possible that the development of an effective dsRNA delivery system could solve this issue. This could generate an effective and highly targeted method for reducing the virulence of *Z. tritici* infections through gene knockdowns, although this would rely on knowledge of which genetic sequences are necessary to the infection process across a broad range of *Z. tritici* isolates.

Fuller understanding of the interactions involved in this pathosystem may also allow effective interventions to be made at later stages in disease development, for example by neutralising effectors within the plant. Another option for preventing epidemics could be resistance priming, using effectors from avirulent *Z. tritici* strains to activate defence responses in crops when virulent *Z. tritici* infestations break out in the area. The practicality of this would likely depend on both the ease with which such avirulence factors could be produced (e.g. using transgenic yeasts) and stored for transport, and on whether a concentration could be found for spraying that activated host defences sufficiently to activate plant defences without causing too much yield loss in uninfected plants. Finally, if an examination of leaf surface-*Z. tritici* interactions reveals tendencies for particular leaf surface patterns or structures to be associated with increased resistance, this could be bred for, using basic microscopy to examine seedling leaf surfaces. Such breeding could result in the production of wheat with higher non-specific resistance to *Z. tritici* (which is currently badly lacking) or which are better protected by resistances which may appear weak or limited in other wheat lines. This method, however, would likely be labour intensive and the practicality or efficacy of breeding for such features is difficult to predict.

Chapter 5 – General discussion

Completion of Thesis aims and objectives

This study generated significant new data and tools that will be of both scientific and practical use as the field progresses. The data produced regarding the effectiveness of disease resistance genes *Stb1*-*Stb19* from the large bioassays and quasi-NIL disease phenotyping tests conducted as part of this work will form the basis for comparisons that will allow scientists to compare and contrast the relative effectiveness of each resistance gene against *Z. tritici* populations present at different times and in different countries. It will also help to guide UK wheat breeders to focus their efforts on the most effective *Stb* genes. The production of such a dataset fulfils the aims of this project, and will generate additional benefits when combined with data generated using *Z. tritici* isolate libraries collected in other areas of the world going forward. Unfortunately this experiment was designed to provide general overall insights into the broad efficacy of each Stb resistance gene studied, and the data generated cannot be considered statistically significant for any individual *Z. tritici* isolate-wheat line interaction – individual interactions of interest to other researchers would therefore require additional testing with more focused screens to confirm. Additionally, a minimal number of wheat lines containing known major resistance genes was tested here – however, moderately to highly aggressive *Z. tritici* isolates selected from the library used here could also be tested against broader ranges of elite wheat lines with less well defined *Stb* resistance genes to identify potentially novel resistance QTLs in future work. Finally, this work used an effectively randomly selected set of *Stb6 Z. tritici* isolates from across the UK, under the assumption that the high genetic diversity of this pathogen would result in most local UK populations showing similar phenotypic diversity to the overall population. However, it is possible that more focused regional tests conducted on isolates selected from specific subsections of the UK where *Z. tritici* is a major issue could identify regional tendencies that were not observed in this work, with potential significance for the wheat lines recommended for breeding programs aimed at producing new varieties for these areas.

The newly developed KASP markers validated using segregating wheat populations will provide tools for fundamental research aimed at isolating *Stb* genes of interest. These tools will also be useful for commercial breeding projects aiming to use these resistance genes in elite lines (especially if the pyramiding of multiple resistance genes is desired). Information gathered on the effects of each *Stb* resistance gene in lines such as Synthetic M3 (in which *Stb16q* was shown to be of highest importance) and Kavkaz-K4500 (in which *Stb10* was shown to be of high importance) will help to more efficiently direct the future efforts of UK wheat breeders. The successful testing of many KASP markers, along with the information gathered regarding the relative

importance of these resistances, fulfils the aims of this project. This work could be fruitfully continued for the remaining *Stb* resistance genes tested in Tidd *et al.* (2023). Future research may also be able to extract further benefits from this work using larger populations and numbers of KASP markers to more accurately map the locations of known *Stb* resistance genes that have not yet been cloned, which unfortunately was not possible in this project due to limitations in the space and manpower available and the failure of a number of the KASP markers used. The identification of the exact genomic locations and sequences of such genes could then be used to produce superior markers using SNPs within the coding sequences of each gene of interest, which could be guaranteed to consistently follow the resistant allele regardless of any crossing over. This work could be further improved through the use of plants grown to a broader variety of ages before inoculation, particularly in cases where the effects of multiple resistance genes within a line were being compared. This is clearly demonstrated in the case of known adult plant resistances such as *Stb17*, the resistance of which was likely underestimated in these results due to the fact that space and manpower limitations, together with the need to keep data comparable across all tested lines and to data collected in (Tidd *et al.*, 2023), required this project to be constrained to measurements of young seedling plants.

The fluorescent *Z. tritici* strains produced in the final stages of this project will enable future research into the mechanisms by which these resistance genes operate, as well as into the ways in which different *Z. tritici* strains interact with other relevant physiological leaf properties. Although the preliminary testing performed here produced little high quality data relating to the mechanisms utilised by the *Stb5* gene, the success of these research tools and the refinements made for future work based on the results presented here will enable further research into such disease responses, fulfilling the aims of this project. The data collected in relation to this area of the project could be significantly improved in future work with fewer limitations on available time and resources, particularly in regards to confirming the transformations made and their stability. Genomic sequencing of the strains produced here could help to identify the genomic locations into which insertions have occurred, allowing verification that they have not damaged the functioning of important genes or introgressed into disposable accessory chromosomes or unstable areas of the genome which may be lost in subsequent generations. Re-plating these strains through a number of generations would also help to ensure that the transformations were stable. Improvements to the ability to account for background autofluorescence in images or the identification of fluorophores that do not overlap as extensively in their emission spectra with background autofluorescence as mCherry in particular

would also enable far better data to be extracted from future repeats of this work.

The knowledge and data obtained in this study is intended to enable breeders to focus their efforts on pyramiding resistance genes most likely to gain in durability when present together. Overall, these scientific advancements and the practical benefits in improved disease resistance in elite wheat varieties will help to secure future crop yields from the damaging effects of STB disease in Europe and worldwide, although further work will be required to verify the conclusions of this work with regards to non-UK *Z. tritici* populations, as only UK isolates were utilised here.

Additionally, it would be beneficial to confirm the conclusions of this work experimentally under field conditions. Although operating in glasshouse systems is more feasible for experiments on the scale described here, such testing necessarily uses *Z. tritici* isolates that are both less diverse than, and several generations removed from, current field populations. This may lead to the isolates used here being less virulent against certain *Stb* resistance genes than more modern field populations, particularly when considering newer *Stb* resistance genes. Furthermore, to efficiently test the hypotheses addressed in this study, wheat lines were inoculated with only one *Z. tritici* isolate at a time throughout the work described in chapters two and three – this is not likely to be realistic under field conditions, and as discussed in chapter four, co-infections with many *Z. tritici* isolates may have very different virulence profiles than most of their component isolates alone. Therefore successful field tests of the efficacy of *Stb* resistance genes of interests have the potential to add significant and valuable context to these results.

Advances in STB resistance should ideally be combined in elite wheat lines with effective and durable resistance genes to other common wheat pathogens (e.g. rusts (Ellis *et al.*, 2014)). This will help to minimise wheat losses due to disease and other pests in general, making it easier to increase crop production without losing large proportions of the yield to diseases that can spread easily through densely packed monocultures. Data gathered during this project will help to direct UK breeders towards the most broadly effective *Stb* resistance genes by identifying those that remain effective against UK *Z. tritici* populations (e.g. *Stb5* and *Stb19*) through large bioassay screens. This data will also help to separate out the *Stb* genes that were most effective in wheat lines containing *Stb* gene pyramids, through the testing of segregating populations and quasi-NILs (e.g. the identification of the significant loss of resistance in Kavkaz-K4500 when *Stb10* is not present, illustrating that this resistance gene is highly important in this pathosystem).

Unfortunately, breeding programmes have limited capacity in the number of traits they can effectively breed for (especially if desirable and undesirable traits are linked), as breeding for more features requires larger breeding populations. Genetic markers such as the KASP markers developed here can help to alleviate this burden by reducing the amount of phenotyping that needs to be done, but they are still limited by the genetic diversity and inheritance patterns in breeding populations.

Given the wide array of challenges faced by modern cropping systems, it would be desirable to be able to more quickly introduce new major resistance genes and other genes that improve wheat resistance to biotic and abiotic challenges directly into elite lines. This could be achieved using genetic modifications if the political climate became more favourable to such technology. However, such techniques require a detailed understanding of the genetic elements underlying a resistance, and it is likely that only cloned genes could be effectively transferred between lines or species (as entire chromosome regions known to contain such genes would be too large). Efforts to clone such resistance genes will benefit from an abundance of high throughput markers to enable large genetic mapping studies to be performed, as well as from the identification of specific *Z. tritici* strains virulent against suitable susceptible parents but avirulent against varieties containing the corresponding resistance gene of interest. Such isolates can be used to screen segregating populations in which each initial hybrid plant is produced from one susceptible and one resistant parent in order to analyse the inheritance patterns of R genes, and to narrow down lists of candidate genes for R gene isolation by examining their co-segregation with known reliable markers.

Determining the genomic location of genes of interest will initially require the narrowing down of candidate physical genome locations for many *Stb* resistance genes. This will be done using large segregating populations in which the segregation of resistance phenotypes and genetic markers can be compared, and thus the genetic distances (in centimorgans) between known markers and resistance genes can be estimated. Previously determined values for wheat chromosomes or estimates developed from examining the segregation of markers at known genomic locations can then be used to convert these centimorgan measurements into physical genomic distances in base pairs. With larger test populations, the KASP markers tested in this project could be useful for this purpose. Sets of likely candidate genes could then be selected based on the nature of previously known *Stb* or other resistance genes – for example, the identification of *Stb6* as a Wall Associated Kinase (WAK) encoding gene (Saintenac *et al.*, 2018) indicates that other appropriately positioned WAK genes are likely to be good candidates. These candidate genes can be conveniently

studied using modern knock-down techniques such as Virus Induced Gene Silencing (VIGS), which delivers double stranded RNA to plant cells using viral vectors to cause the temporary knockdown of genes with a matching sequence through the endogenous DICER and RISC complexes (Ramegowda *et al.*, 2014). A *Z. tritici* isolate known to be avirulent against wheat expressing the resistance gene of interest can then be used to phenotype treated and control plants, with a loss of resistance in treated plants indicating that the candidate gene that has been silenced likely does represent the bona fide *Stb* resistance gene. The more laborious process of cloning this gene and confirming its identity through genetic complementation studies can then take place.

Recent evidence (Battache *et al.*, 2022) suggests that the leaf surface may be a major site for disease resistance gene activity. *Stb6, Stb9* and *Stb16q* were all suggested to operate through early detection of the pathogen, leading to the closure of leaf stomata and the prevention of fungal disease and hence infection. This was determined primarily through confocal microscopic analysis of the leaf surface, supported by observations of the increased virulence of pathogen isolates when infiltrated into leaves or inoculated onto wounded leaves. The fact that this resistance mechanism seems common to at least three *Stb* genes suggests that it may also be responsible the resistance provided by others that operate through as-yet unknown mechanisms. The fluorescent *Z. tritici* strains produced through the work described in this Thesis will enable the kind of detailed experiment conducted by (Battache et al., 2022) to be conducted for several other *Stb* genes of interest, due to the range of virulence profiles available in these fluorescent strains.

Co-inoculating with combinations of virulent and avirulent *Z. tritici* strains could also help to determine whether recognition of an avirulence effector could, in some cases, trigger a resistance mechanism effective against not only avirulent but also co-inoculated virulent strains (as the rate of stomatal closure would be similar to single avirulent inoculation rates, preventing both strains from entering the leaf). Alternatively, such co-inoculation will also reveal if in other cases virulent *Z. tritici* strains could suppress disease resistance responses triggered following recognition of avirulence effectors (which would prevent stomatal closure). The tools produced in this study include *Z. tritici* strains both virulent and avirulent against wheat lines containing *Stb* resistance genes of interest, with each expressing either GFP or mCherry fluorophores. These tools will enable future research to differentiate between virulent and avirulent co-inoculated strains under a confocal microscope in such co-inoculation experiments.

Even in wheat lines with *Stb* resistance genes known to operate partially through stomatal closure, a few fungal hyphae even of avirulent strains still appear to penetrate the leaf surface without

generating major infections. This suggests additional defence mechanisms operating in the apoplast in these resistant host genotypes which will be more difficult to characterise. Once *Stb* resistance gene sequences are known, quantitative (or “real time”) PCRs targeting mRNA coding for these genes could be used to identify individual leaf cells or areas where these resistance genes seem most highly expressed during infection. Transiently or constitutively expressed versions of these resistance genes containing fluorophores could also help to precisely monitor the cellular locations of the proteins produced (although the finer details of such positioning may be disrupted by the presence of a fluorophore in some cases, suggesting that the testing of multiple alternative fluorophores for each such *Stb* protein may be necessary to ensure that observations are reliable).

Investigation of these disease resistance mechanisms will benefit greatly from the increased availability of data and tools related to this pathosystem. The benefits of the markers, *Z. tritici* virulence databases and fluorescent *Z. tritici* strains produced here have already been discussed. However, the wealth of genomic information available for multiple genotypes of the wheat host is also necessary for identifying candidate *Stb* resistance genes for attempted cloning, as well as being extremely directly helpful to breeders (Keller *et al.*, 2018). Improvements to reverse genetics techniques (e.g. VIGS and CRISPR/Cas9 (Ramegowda *et al.*, 2014; Shen *et al.*, 2014) for the knock down or knock out of host genes) will provide additional tools for speeding up the cloning and conformation of the identity of *Stb* genes. Advancements from continuing research into other pathosystems may also produce information that could help to guide this field forward. For example, the discovery of the identity of resistance genes in other pathosystems as WAKs (e.g. Hu *et al.*, 2017) helped to prioritise WAK genes as *Stb* resistance gene candidates, leading to the identification of *Stb6* as a WAK gene (Saintenac *et al.*, 2018).

Overall, the continued development of STB resistance in UK and European wheat forms an important part of the wider goal of protecting and improving yields to feed a growing global population from a reduced land area whilst suffering from both the biotic and abiotic consequences of climate change. This effort will require improvements to wheat resilience against many diseases, along with increases in baseline yield and improved resistance to damage from abiotic factors such as cold, heat or drought. It will be vital that scientists are involved throughout this process, to advise on the optimal combinations of resistance genes in elite wheat varieties, and the optimal patterns of deployment of these varieties, for example in rotations, mixtures or mosaics, to maximise the effectiveness and durability of these resistance genes. The maintenance of landraces and the development of synthetic wheat lines may allow the identification of new *Stb* genes,

which could provide breeders with new disease resistance options. Predictions from climate scientists will also be vital for allowing breeders to future-proof new varieties against the expected effects of climate change.

Finally, genetic research could dramatically increase the rate of improvement of wheat and other crops if the political will existed to permit genetically modified crops to be utilised to their full potential. Such techniques would be especially beneficial when applied to resistance genes identified from landraces and synthetic wheat lines, which this and other projects have shown to carry some of the most broadly effective novel resistance genes, due to the potential for linkage drag when these lines are added to breeding programs. Additionally, enabling growers to access genetically modified plants would allow for the use of genes from different plant species, including resistances from species which are not hosts to *Z. tritici*. It is likely that this would make large numbers of highly effective and durable new resistance genes available to breeders and growers. All information gathered on the structure of the wheat genome, the location and identity of genes of interest, and the mechanisms by which these genes operate (particularly any identification of other genes that may be necessary to facilitate the resistance provided by *Stb* resistance genes) will contribute usefully towards this goal. It is hoped that the tools and data generated throughout this PhD will aid in the molecular and physiological research required to achieve these objectives, as well as directly aiding UK wheat breeders with the production of new disease resistant lines.

Summary of recommendations for future work

- The genetic markers and datasets of broadly effective *Stb* resistance genes produced here will allow breeders to begin breeding *Z. tritici* resistant wheat lines immediately – ideally these lines should contain resistant gene pyramids to enhance durability.
- The KASP markers produced here and other modern markers in the wheat genome can be used to more precisely map the genomic locations of broadly effective *Stb* resistance genes.
- VIGS and other methods for causing genetic knockdowns and knockouts will allow reverse genetic screens for additional *Stb* resistance genes. The genetic information now available on the wheat genome will help to target such efforts. WAK genes are an obvious early target.
- Genetic modification techniques could theoretically enable breeders to utilise *Stb* resistance genes more quickly and produce more durable gene pyramids once suitably broadly effective Stb resistance genes are identified.
- The cloning of *Stb* resistance genes would also enable qPCR and fluorescent tagging to monitor their protein expression and localisation *in planta* during infections.
- Fluorescent *Z. tritici* strains produced in this Thesis, and other fluorescent strains produced using similar techniques in other work (possibly from isolates identified as useful from datasets produced in this Thesis) successfully complete the intended confocal observation of inoculations and co-inoculations of wheat leaves with the methodological adjustments suggested in this work, in order to determine how broadly utilised and effective leaf surface defence mechanisms such as stomatal closure are in this pathosystem.
- Fluorescent lines produced in this project would also benefit from a transformation stability analysis conducted over several fungal generations to determine the genetic stability of the transformations made. Genetic sequencing to determine the location of the insert in the fungal genome would also help to confirm the utility of these strains as research tools.
- Sequencing the isolates contained in the *Z. tritici* library used in the bioassays presented here would also increase the value of this library as a tool for UK wheat breeders and researchers, who will benefit from having a large number of modern *Z. tritici* isolates of known virulence against a number of wheat lines from which to select inoculum for future experiments and breeding programs. For isolates of particular interest, an expansion of the testing conducted in these bioassays could also help to statistically confirm the virulence patterns against each wheat line seen in this data at the individual isolate level.

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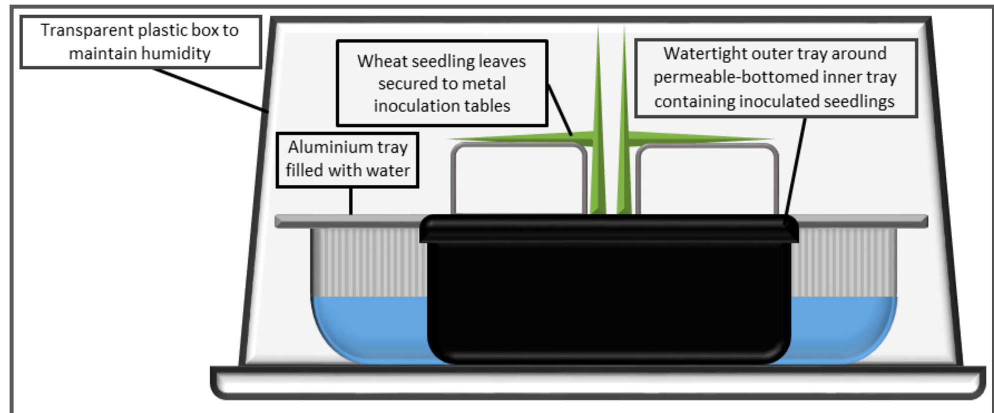
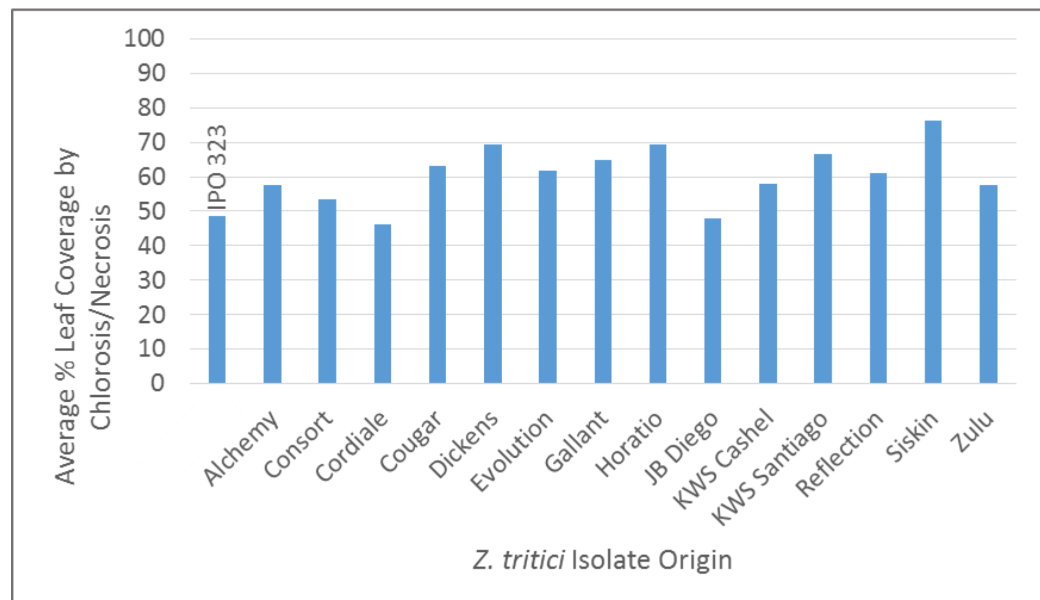
Supplementary Figures

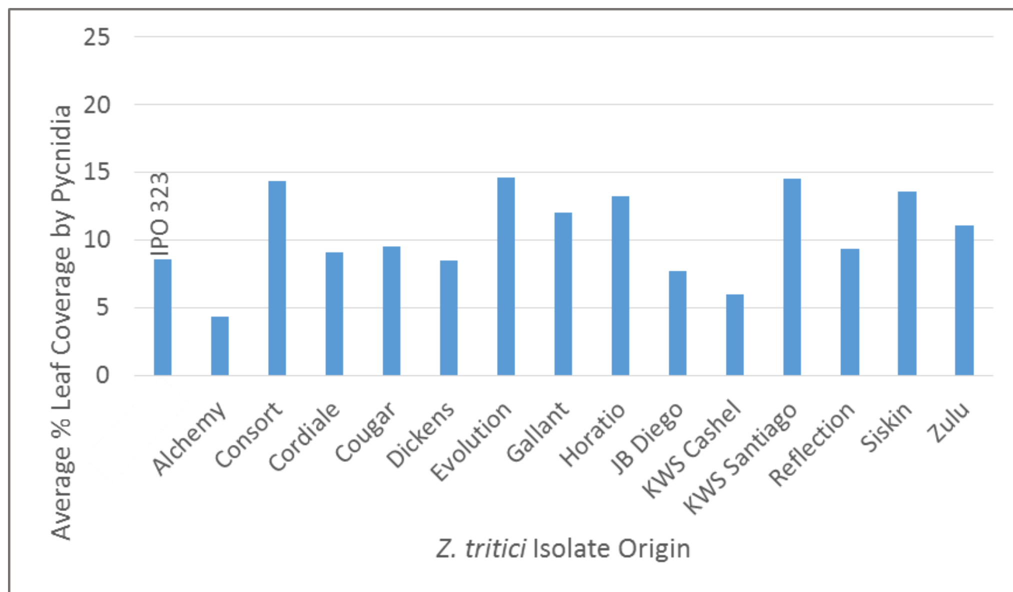
Chapter 2 - A large bioassay identifies Stb resistance genes that provide broad resistance against Septoria tritici blotch disease in the UK- Supplementary Figures



Supplementary Figure 1: Experimental set up for high humidity incubation of seedlings during the first 72 hours post inoculation with *Zymoseptoria tritici*



Supplementary Figure 2: Average percentage of inoculated leaf area covered by necrosis and chlorosis on 17 different wheat genotypes at 28 days post inoculation for *Z. tritici* isolates sourced from different wheat varieties (indicated on the X-axis) or the reference isolate IPO 323. A single-factor ANOVA test shows that the variations between the average symptom coverages caused by *Z. tritici* isolates sourced from the different wheat genotype are significant (p=0.001).



Supplementary Figure 3: Average percentage of inoculated leaf area covered by pycnidia on 17 different wheat genotypes at 28 days post inoculation for *Z. tritici* isolates sourced from different wheat varieties (indicated on the X-axis) or the reference isolate IPO 323. A single-factor ANOVA test shows that the variations between the average symptom coverages caused by *Z. tritici* isolates sourced from the different wheat genotype are not significant (p=0.114).

Supplementary Table 1: Full data showing which wheat genotypes could be classified as resistant or susceptible based on the % leaf area covered by chlorosis/necrosis at 28 days post inoculation with different *Zymoseptoria tritici* isolates. Interactions classified as resistant failed to produce any visible chlorosis or necrosis symptoms across the tested leaves. Missing data is represented with a dash.

	Taichung 29	Riband	KWS Cashel	Synthetic 6X	Synthetic M3	Kavkaz K4500	Tadinia	Estanzuela Federal	Israel 493	TE9111	Bulgaria 88	Veranopolis	Synthetic M6	Tonic	Salamouni	Balance	Lorikeet
IPO 323	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
RResHT-1	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-2	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-
RResHT-3	Susceptible	Susceptible	Susceptible	Susceptible	-	Resistant	Susceptible	Susceptible	Resistant	Susceptible	-	-	-	-	-	Susceptible	-
RResHT-4	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-5	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	-	-	-	-	-	Susceptible	-
RResHT-6	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-7	Susceptible	Susceptible	Susceptible	Susceptible	-	Resistant	Susceptible	Susceptible	Susceptible	Resistant	-	-	-	-	-	Susceptible	-
RResHT-8	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-
RResHT-9	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-10	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-
RResHT-11	Susceptible	Susceptible	Susceptible	-	-	-	-	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-12	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	-	Susceptible	Susceptible	-	-	-	-	-	Susceptible	-
RResHT-13	Susceptible	Susceptible	Susceptible	Susceptible	-	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-14	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	-	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	-
RResHT-15	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	-	-	-	-	Susceptible	-
RResHT-16	Susceptible	Susceptible	Susceptible	Susceptible	-	-	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	-	-	-
RResHT-17	Susceptible	Susceptible	Susceptible	-	-	-	-	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-18	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-19	Susceptible	Susceptible	Susceptible	Susceptible	-	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-20	Susceptible	-	-	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	-
RResHT-21	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	-	-	-	-	-	-	-
RResHT-22	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	-	-	-	-	-	Susceptible	-
RResHT-23	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-24	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
RResHT-25	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Resistant	-	-	-	-	-	-	-
RResHT-26	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	-	-	-	-	-	Susceptible	-
RResHT-27	Susceptible	Susceptible	Susceptible	Susceptible	-	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-28	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-29	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
RResHT-30	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	-	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	-
RResHT-31	Susceptible	Susceptible	Susceptible	Susceptible	-	-	Susceptible	-	-	Susceptible	Susceptible	Susceptible	Susceptible	-	-	-	-
RResHT-32	-	Susceptible	Susceptible	-	-	-	-	-	-	Susceptible	-	-	-	-	-	-	-
RResHT-33	-	Susceptible	Susceptible	Susceptible	-	-	Susceptible	-	-	Susceptible	-	-	-	-	-	-	-
RResHT-34	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant
RResHT-35	-	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	-	-	Resistant	-	-	-	-	-	-	-
RResHT-36	-	Susceptible	Susceptible	Susceptible	-	Resistant	Susceptible	-	-	Resistant	-	-	-	-	-	Susceptible	-
RResHT-37	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
RResHT-38	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
RResHT-39	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
RResHT-41	-	Susceptible	Susceptible	Susceptible	-	Resistant	Susceptible	-	-	Susceptible	-	-	-	-	-	Susceptible	-
RResHT-42	-	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	-	-	-	-	-	-	-	-	Susceptible	-
RResHT-44	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant
RResHT-45	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant
RResHT-46	-	Susceptible	-	-	-	-	-	-	Susceptible	-	-	-	-	-	-	-	-
RResHT-47	-	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	-	Susceptible	-	-	-	-	-	-	Susceptible	-
RResHT-48	-	Susceptible	Susceptible	Resistant	-	Resistant	-	-	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-49	-	Susceptible	-	-	-	-	-	-	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-50	-	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	-	-	-	-	-	-	-	-	Susceptible	-
RResHT-51	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant
RResHT-52	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant
RResHT-53	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant
RResHT-54	-	Susceptible	Susceptible	Resistant	-	-	-	-	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-55	-	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-	-	-	-
RResHT-56	-	Susceptible	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RResHT-57	-	Susceptible	-	-	-	-	-	-	Susceptible	-	-	-	-	-	-	-	-
RResHT-58	-	Susceptible	-	-	-	-	-	-	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-60	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
RResHT-62	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
RResHT-63	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
RResHT-64	Susceptible	-	-	Susceptible	Susceptible	-	Susceptible	-	-	Resistant	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	-
RResHT-65	-	-	-	Susceptible	-	-	Susceptible	-	-	Susceptible	Susceptible	Susceptible	Resistant	-	-	-	-
RResHT-66	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible
RResHT-67	Susceptible	Susceptible	Susceptible	Susceptible	-	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible
RResHT-68	-	Susceptible	Susceptible	-	-	-	-	-	-	-	-	-	Susceptible	-	-	-	Susceptible
RResHT-69	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
RResHT-70	-	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible
RResHT-71	-	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible	-	Susceptible
RResHT-72	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	-	-	-	-	Susceptible	-
RResHT-73	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
RResHT-74	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	-	-	-	-	Susceptible	-
RResHT-75	Susceptible	Susceptible	Susceptible	-	-	-	-	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-76	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible	-	-	Susceptible	-	-	-	Resistant

RResHT-78	Susceptible	Susceptible	Susceptible	-	-	-	-	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-79	Susceptible	Susceptible	Susceptible	-	-	-	-	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-80	Susceptible	Susceptible	Susceptible	-	-	-	-	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-82	Susceptible	Susceptible	Susceptible	-	-	-	-	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-83	Susceptible	Susceptible	Susceptible	-	-	-	-	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-84	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-85	Susceptible	Susceptible	Susceptible	Resistant	-	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-86	Susceptible	Susceptible	Susceptible	-	-	-	-	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-87	Susceptible	Susceptible	Susceptible	-	-	-	-	Susceptible	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-91	-	Susceptible	Susceptible	Susceptible	-	-	Susceptible	-	Susceptible	Susceptible	-	-	-	-	-	-	-
RResHT-92	-	Susceptible	Susceptible	Susceptible	-	-	Susceptible	-	-	Susceptible	-	-	-	-	-	-	-
RResHT-93	-	Susceptible	Susceptible	-	-	-	-	-	-	Susceptible	-	-	-	-	-	-	-
RResHT-94	-	Susceptible	-	-	-	-	-	-	-	Susceptible	-	-	-	-	-	-	-
RResHT-95	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible
RResHT-96	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Resistant
RResHT-97	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible
RResHT-98	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
RResHT-99	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant
RResHT-100	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Susceptible
RResHT-101	-	Susceptible	Susceptible	-	-	-	Susceptible	-	Susceptible	-	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible
RResHT-102	-	-	Susceptible	-	Susceptible	Susceptible	-	-	-	-	-	-	Susceptible	-	-	-	Resistant

Supplementary Table 2: Full data showing which wheat genotypes could be classified as resistant or susceptible based on the % leaf area covered by pycnidia at 28 days post inoculation with different *Zymoseptoria tritici* isolates. Interactions classified as resistant failed to produce any visible pycnidia across the tested leaves. Missing data is represented with a dash.

Z. tritici isolate	Taichung 29	Riband	KWS Cashel	Synthetic 6X	Synthetic M3	Kavkaz K4500	Tadinia	Estanzuela Federal	Israel 493	TE9111	Bulgaria 88	Veranopolis	Synthetic M6	Tonic	Salamouni	Balance	Lorikeet
IPO 323	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Resistant	Resistant
RResHT-1	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-2	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Susceptible	Susceptible	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Resistant	-
RResHT-3	Susceptible	Susceptible	Susceptible	Susceptible	-	Resistant	Resistant	Resistant	Resistant	Resistant	-	-	-	-	-	Resistant	-
RResHT-4	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-5	Susceptible	Susceptible	Susceptible	Resistant	-	Resistant	Resistant	Susceptible	Resistant	Resistant	-	-	-	-	-	Resistant	-
RResHT-6	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-7	Susceptible	Susceptible	Susceptible	Resistant	-	Resistant	Resistant	Susceptible	Resistant	Resistant	-	-	-	-	-	Resistant	-
RResHT-8	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Susceptible	Susceptible	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant	Susceptible	Resistant	-
RResHT-9	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Resistant	-	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-10	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Resistant	Resistant	-
RResHT-11	Susceptible	Susceptible	Susceptible	-	-	-	-	Susceptible	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-12	Susceptible	Susceptible	Susceptible	Resistant	-	Resistant	Resistant	-	Resistant	Resistant	-	-	-	-	-	Resistant	-
RResHT-13	Susceptible	Susceptible	Susceptible	Resistant	-	Resistant	Resistant	Resistant	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-14	Susceptible	Susceptible	Susceptible	Resistant	Resistant	-	Resistant	Susceptible	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant	-	-
RResHT-15	Susceptible	Susceptible	Susceptible	Resistant	-	Resistant	Resistant	Susceptible	Resistant	Resistant	-	-	-	-	-	Resistant	-
RResHT-16	Susceptible	Susceptible	Susceptible	Resistant	-	-	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Resistant	Susceptible	-	-	-	-
RResHT-17	Susceptible	Susceptible	Resistant	-	-	-	-	Susceptible	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-18	Susceptible	Susceptible	Susceptible	Resistant	-	Resistant	Susceptible	Susceptible	Resistant	Susceptible	-	-	-	-	-	-	-
RResHT-19	Susceptible	Resistant	Susceptible	Resistant	-	Resistant	Susceptible	Resistant	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-20	Susceptible	-	-	Resistant	Resistant	Resistant	Resistant	Susceptible	-	Resistant	Resistant	Resistant	Susceptible	Susceptible	-	Susceptible	-
RResHT-21	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-22	Susceptible	Susceptible	Susceptible	Resistant	-	Resistant	Susceptible	Susceptible	Resistant	Susceptible	-	-	-	-	-	Resistant	-
RResHT-23	Susceptible	Susceptible	Susceptible	Resistant	-	Resistant	Susceptible	Susceptible	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-24	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Resistant
RResHT-25	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Susceptible	Susceptible	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-26	Resistant	Susceptible	Susceptible	Resistant	-	Resistant	Susceptible	Resistant	Resistant	Resistant	-	-	-	-	-	Susceptible	-
RResHT-27	Resistant	Susceptible	Susceptible	Resistant	-	Resistant	Susceptible	Resistant	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-28	Resistant	Susceptible	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-29	Resistant	Susceptible	Susceptible	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant	Resistant
RResHT-30	Resistant	Susceptible	Susceptible	Resistant	Resistant	Resistant	Resistant	-	-	Resistant	Susceptible	Susceptible	Susceptible	-	Resistant	Resistant	-
RResHT-31	Susceptible	Susceptible	Susceptible	Resistant	-	-	Resistant	-	-	Resistant	Susceptible	Resistant	Susceptible	-	-	-	-
RResHT-32	-	Susceptible	Susceptible	-	-	-	-	-	-	Resistant	-	-	-	-	-	-	-
RResHT-33	-	Susceptible	Susceptible	Resistant	-	-	Susceptible	-	-	Resistant	-	-	-	-	-	-	-
RResHT-34	Susceptible	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
RResHT-35	-	Susceptible	Susceptible	Resistant	Resistant	Resistant	Susceptible	-	-	Resistant	-	-	-	-	-	-	-
RResHT-36	-	Susceptible	Susceptible	Resistant	-	Resistant	Susceptible	-	-	Resistant	-	-	-	-	-	Resistant	-
RResHT-37	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Resistant	Resistant
RResHT-38	Susceptible	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant	Resistant
RResHT-39	Resistant	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
RResHT-41	-	Susceptible	Susceptible	Resistant	-	Resistant	Susceptible	-	-	Resistant	-	-	-	-	-	Resistant	-
RResHT-42	-	Susceptible	Susceptible	Resistant	-	Resistant	Susceptible	-	-	-	-	-	-	-	-	Resistant	-
RResHT-44	Susceptible	Resistant	Susceptible	-	Resistant	Susceptible	Susceptible	Susceptible	-	Susceptible	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Resistant
RResHT-45	Susceptible	Resistant	Susceptible	Susceptible	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant
RResHT-46	-	Susceptible	-	-	-	-	-	-	Resistant	-	-	-	-	-	-	-	-
RResHT-47	-	Susceptible	Susceptible	Resistant	-	Resistant	Susceptible	-	Resistant	-	-	-	-	-	-	Resistant	-
RResHT-48	-	Resistant	Susceptible	Resistant	-	Resistant	-	-	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-49	-	Susceptible	-	-	-	-	-	-	Resistant	Resistant	-	-	-	-	-	-	-

RResHT-50	-	Susceptible	Susceptible	Resistant	-	Resistant	Susceptible	-	-	-	-	-	-	-	-	Resistant	-
RResHT-51	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Resistant
RResHT-52	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Resistant	Resistant
RResHT-53	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	-	Susceptible	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant
RResHT-54	-	Susceptible	Susceptible	Resistant	-	-	-	-	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-55	-	Susceptible	Susceptible	Resistant	Resistant	Resistant	Susceptible	-	-	-	-	-	-	-	-	-	-
RResHT-56	-	Susceptible	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RResHT-57	-	Susceptible	-	-	-	-	-	-	Resistant	-	-	-	-	-	-	-	-
RResHT-58	-	Susceptible	-	-	-	-	-	-	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-60	Resistant	Susceptible	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant
RResHT-62	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant
RResHT-63	Resistant	Susceptible	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant
RResHT-64	Resistant	-	-	Resistant	Resistant	-	Susceptible	-	-	Resistant	Resistant	Resistant	Resistant	-	Resistant	Resistant	-
RResHT-65	-	-	-	Resistant	-	-	Susceptible	-	-	Resistant	Resistant	Susceptible	Resistant	-	-	-	-
RResHT-66	Resistant	Susceptible	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	-	Resistant	Resistant	Resistant
RResHT-67	Resistant	Susceptible	Resistant	Resistant	-	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	-	Resistant	Resistant	Resistant
RResHT-68	-	Resistant	Resistant	-	-	-	-	-	-	-	-	-	Resistant	-	-	-	Resistant
RResHT-69	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Resistant
RResHT-70	-	Susceptible	Susceptible	-	Resistant	Resistant	Susceptible	-	Resistant	Susceptible	Resistant	Susceptible	Susceptible	-	Resistant	Resistant	Resistant
RResHT-71	-	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant	-	Resistant	Resistant	Resistant	-	Susceptible	Resistant	Resistant	-	Resistant
RResHT-72	Resistant	Susceptible	Susceptible	Resistant	-	Resistant	Susceptible	Susceptible	Resistant	Susceptible	-	-	-	-	-	Resistant	-
RResHT-73	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Resistant
RResHT-74	Susceptible	Susceptible	Susceptible	Resistant	-	Resistant	Susceptible	Resistant	Susceptible	Resistant	-	-	-	-	-	Susceptible	-
RResHT-75	Resistant	Susceptible	Susceptible	-	-	-	-	Susceptible	Resistant	Susceptible	-	-	-	-	-	-	-
RResHT-76	Susceptible	Susceptible	Susceptible	-	Resistant	Resistant	-	Susceptible	Resistant	Resistant	-	-	Susceptible	-	-	-	Resistant
RResHT-78	Resistant	Susceptible	Susceptible	-	-	-	-	Susceptible	Susceptible	Resistant	-	-	-	-	-	-	-
RResHT-79	Resistant	Susceptible	Susceptible	-	-	-	-	Susceptible	Susceptible	Resistant	-	-	-	-	-	-	-
RResHT-80	Susceptible	Susceptible	Susceptible	-	-	-	-	Susceptible	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-82	Resistant	Susceptible	Susceptible	-	-	-	-	Susceptible	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-83	Resistant	Susceptible	Susceptible	-	-	-	-	Susceptible	Resistant	Susceptible	-	-	-	-	-	-	-
RResHT-84	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-85	Susceptible	Susceptible	Susceptible	Resistant	-	Resistant	Susceptible	Susceptible	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-86	Susceptible	Susceptible	Susceptible	-	-	-	-	Susceptible	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-87	Susceptible	Susceptible	Susceptible	-	-	-	-	Susceptible	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-91	-	Susceptible	Susceptible	Resistant	-	-	Susceptible	-	Resistant	Resistant	-	-	-	-	-	-	-
RResHT-92	-	Susceptible	Susceptible	Resistant	-	-	Susceptible	-	-	Resistant	-	-	-	-	-	-	-
RResHT-93	-	Susceptible	Susceptible	-	-	-	-	-	-	Resistant	-	-	-	-	-	-	-
RResHT-94	-	Susceptible	-	-	-	-	-	-	-	Resistant	-	-	-	-	-	-	-
RResHT-95	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	-	Resistant	Resistant
RResHT-96	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	-	Susceptible	Resistant
RResHT-97	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	-	Resistant	Susceptible	Resistant
RResHT-98	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Susceptible	Susceptible	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
RResHT-99	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Resistant
RResHT-100	Susceptible	Resistant	Susceptible	Resistant	-	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Susceptible	Resistant	-	Resistant	Resistant
RResHT-101	-	Susceptible	Susceptible	-	-	-	Susceptible	-	Resistant	-	Susceptible	Resistant	Resistant	Resistant	Resistant	-	Resistant
RResHT-102	-	-	Susceptible	-	Resistant	Resistant	-	-	-	-	-	-	Susceptible	-	-	-	Resistant

Supplementary Table 3: *Zymoseptoria tritici* isolates used in this study together with their collection dates and the wheat varieties from which they were sampled.

***Z. tritici* isolate**	**Year collected**	**Wheat variety from which isolate was sourced**
RResHT-1	2015	Consort
RResHT-2	2015	Gallant
RResHT-3	2015	KWS Cashel
RResHT-4	2015	KWS Cashel
RResHT-5	2015	KWS Cashel
RResHT-6	2016	Consort
RResHT-7	2016	Consort
RResHT-8	2016	Cougar
RResHT-9	2016	Consort
RResHT-10	2015	Gallant
RResHT-11	2015	Zulu
RResHT-12	2015	Cougar
RResHT-13	2015	Horatio
RResHT-14	2015	Cougar
RResHT-15	2015	Cougar
RResHT-16	2016	Consort
RResHT-17	2015	Cougar
RResHT-18	2015	KWS Santiago
RResHT-19	2015	JB Diego
RResHT-20	2015	Cougar
RResHT-21	2015	Cougar
RResHT-22	2015	KWS Cashel
RResHT-23	2016	Consort
RResHT-24	2016	Consort
RResHT-25	2016	Consort
RResHT-26	2015	Cougar
RResHT-27	2015	Gallant
RResHT-28	2015	Cougar
RResHT-29	2016	Cordiale
RResHT-30	2016	Cordiale
RResHT-31	2016	Cordiale
RResHT-32	2016	Cordiale
RResHT-33	2016	Cordiale
RResHT-34	2016	Cordiale
RResHT-35	2016	Cordiale
RResHT-36	2016	Cordiale
RResHT-37	2016	Cordiale
RResHT-38	2016	Cordiale
RResHT-39	2016	Cordiale
RResHT-40	2016	Dickens

RResHT-41	2016	Dickens
RResHT-42	2016	Dickens
RResHT-43	2016	Dickens
RResHT-44	2016	Dickens
RResHT-45	2016	KWS Santiago
RResHT-46	2016	KWS Santiago
RResHT-47	2016	Alchemy
RResHT-48	2016	Cordiale
RResHT-49	2015	KWS Cashel
RResHT-50	2016	Dickens
RResHT-51	2016	Dickens
RResHT-52	2016	Dickens
RResHT-53	2015	KWS Cashel
RResHT-54	2016	Dickens
RResHT-55	2016	Dickens
RResHT-56	2016	Dickens
RResHT-57	2016	Dickens
RResHT-58	2016	Dickens
RResHT-59	2016	Dickens
RResHT-60	2016	Dickens
RResHT-61	2016	Dickens
RResHT-62	2016	Dickens
RResHT-63	2016	Dickens
RResHT-64	2015	KWS Cashel
RResHT-65	2015	KWS Cashel
RResHT-66	2015	KWS Cashel
RResHT-67	2015	KWS Cashel
RResHT-68	2015	KWS Cashel
RResHT-69	2015	KWS Cashel
RResHT-70	2015	KWS Cashel
RResHT-71	2015	KWS Cashel
RResHT-72	2015	Dickens
RResHT-73	2016	Reflection
RResHT-74	2017	Siskin
RResHT-75	2017	Siskin
RResHT-76	2017	Siskin
RResHT-77	2017	Siskin
RResHT-78	2017	Siskin
RResHT-79	2017	Siskin
RResHT-80	2017	Siskin
RResHT-81	2017	Siskin
RResHT-82	2017	Siskin
RResHT-83	2017	Siskin
RResHT-84	2017	Siskin
RResHT-85	2015	Cougar

RResHT-86	2015	Cougar
RResHT-87	2015	Cougar
RResHT-88	2015	Cougar
RResHT-89	2015	Cougar
RResHT-90	2016	Evolution
RResHT-91	2016	Evolution
RResHT-92	2016	Evolution
RResHT-93	2016	JB Diego
RResHT-94	2016	Zulu
RResHT-95	2016	Dickens
RResHT-96	2016	Dickens
RResHT-97	2016	Consort
RResHT-98	2016	Reflection
RResHT-99	2016	Reflection
RResHT-100	2016	Reflection
RResHT-101	2016	Reflection
RResHT-102	2017	Siskin
RResHT-103	2017	Siskin
RResHT-104	2016	Evolution

Supplementary Table 4: Table showing the number of *Z. tritici* isolates in the test set originally isolated from each wheat variety in the field (along with the standard control isolate IPO 323).

Wheat variety from which isolate was sourced	No. isolates
Alchemy	1
Consort	9
Cordiale	12
Cougar	14
Dickens	21
Evolution	4
Gallant	3
Horatio	1
JB Diego	2
KWS Cashel	14
KWS Santiago	3
Reflection	5
Siskin	13
Zulu	2

Chapter 3 - Controlled environment tests on wheat Quasi-NIL lines reveal optimal candidates for Stb disease resistance gene pyramids effective against Zymoseptoria tritici - Supplementary Figures

Supplementary Table 1: The sequences used in each successful KASP marker set (showing statistically significant correlation with at least one infection phenotype score), with the genetic regions they were designed to target. KASP markers designed using the WAK promotome capture data show the SNPs targeted by these markers as [Genotype 1/Genotype 2]. For KASP markers developed from Axiom array data, target SNPs are given using standard IUPAC notation. The markers labelled with the prefix cfn are not included here as these markers have been reported by previous papers (Saintenac *et al.*, 2018; Saintenac *et al.*, 2021).

KASP marker designation	Developed From	*Stb* gene targeted	Position of KASP marker target	Targeted genomic sequence	Fluorescent marker 1	Fluorescent marker 2	Common reverse (with quencher)
7D_120312071_Stb5_K	Rothamsted Research WAK Promotome Data	*Stb5*	Position 120312071, Chromosome 7D	ATATCCTATAAAACCAGGTTCAGAAACGGTTTCGATCGAAAATTTTCCTAACCACTTGCACCCTTATTTTTCTACAGCAATTTAGGATTAGTTAATGATGTGCAGATTAGGTTAATAAGATTGATAACAAATTCTAGCAACTGATATTGGCAAAGCGGTAGAACGAAATACGTCTCCATCATGCTATCGATTACATCAAC[A/G]CATTAGACATGGAGAGATTACTGCAGCACCTTATCTTCACGTCTTCTGACTTTTTTGTCTTTTCCCGTGCACACCATCGTCCACCATTTCGGGCCTCTTTGATTCGTAGGATTTTGAAAACGTAGGAATGGGAAAAGTATAGGATTGGAGTGGCATGCCCACTTGAATCCTATAGGATTAACAAGGAGTGTTTGATGGCA	GAAGGTGACCAAGTTCATGCTCCATCATGCTATCGATTACATCAACA	GAAGGTCGGAGTCAACGGATTCCATCATGCTATCGATTACATCAACG	GACGTGAAGATAAGGTGCTGCAGTA

EC_Stb5_7D_9 6724451_k	Rothamsted Research WAK Promotome Data	*Stb5*	Position 96724451, Chromosome 7D	CCTTGGTTTCAACACTGAGGGAAATA CCTACCGTCGCTGTGCTGCATCATCC CTTCCTCTCTGGGGAAATACCGACGT AGTCCTAGCAGACATCAAGCGGCGC CACGCGGCGTTCACACCGCAGCGAT GGAGGAGGCGTCCGTCGGACCACC GGGTTTCTGGGCGAGTCGACGTGGG ACCCCTTC[G/A]TCAGTCCTACATGC GGGCGTGCCCGGACGCCCCCATATC CGTCCCATATTGGGCTAGATATGTGG GGTGCCGGTCAGCCGGGCATTTGAG GCCCGTTTGAGGGGGTCGTCTGGGT CATAAAAACGTGACTGGTCAGTGACC GGGTGGCCCGCCCGGGCGTATGTG ACGCGTTTGGGGCACCCGGCTGTAG ATGCTCT	GAAGGTGAC CAAGTTCAT GCTCGCCCG CATGTAGGA CTGAC	GAAGGTCGG AGTCAACGG ATTACGCCC GCATGTAGG ACTGAT	CGAGTCGA CGTGGGA CCCCTT
EC_Stb5_7D_9 7759874_k	Rothamsted Research WAK Promotome Data	*Stb5*	Position 97759874, Chromosome 7D	GCCAGCACGTCCGCGCCCACTGCCA TCATCGAGTAGAAGTGGAACATGGG AGGTCACCGCTGTGCTTGGGTCCCA TGAAGACCACCACGGAGGCGACGCC GGTGTACATAAGATGTGTCTTGCCTG GTCGCAGGCGACCGTCGTCCCCCGC CCCTCCGGTCGAAGCACATCCCGCA GTGCCACTAAGATGAGCGGTGCTAT CAAACATGGGGAAGCGAGCTGCTCT TTGTG[C/T]TGAAGCATATACCTCCGG GGCTCAAGGCAGTCCAATAGCGGGC TGCCAGACATGGGGGAAGACAATGA AGATCCGCCGTGGTTGGCCATAGAC TAGTCAAGGGTATCCGTGTCATGGG AATGGATATCCGCCGGTGTCGGTCG TAGACTAGTTTTATCTTAGTCCGGTAT ATTTTTAGTTAAAAATGCCCAATATGT AATGAATTCATCTGGTTTATATGTAAT TCGTCGTGTTTGCATGAA	GAAGGTGAC CAAGTTCAT GCTGAAGCG AGCTGCTCT TTGTGC	GAAGGTCGG AGTCAACGG ATTGGAAGC GAGCTGCTC TTTGTGT	GCCTTGAG CCCCGGA GGTATAT

EC_Stb5_7D_96724451_p	Rothamsted Research WAK Promotome Data	*Stb5*	Position 96724451, Chromosome 7D	CCTTGGTTTCAACACTGAGGGAAATACCTACCGTCGCTGTGCTGCATCATCCCTTCCTCTCTGGGGAAATACCGACGTAGTCCTAGCAGACATCAAGCGGCGCCACGCGGCGTTCACACCGCAGCGATGGAGGAGGCGTCCGTCGGACCACCGGGTTTCTGGGCGAGTCGACGTGGGACCCCTTC[G/A]TCAGTCCTACATGCGGGCGTGCCCGGACGCCCCCATATCCGTCCCATATTGGGCTAGATATGTGGGGTGCCGGTCAGCCGGGCATTTGAGGCCCGTTTGAGGGGGTCGTCTGGGTCATAAAAACGTGACTGGTCAGTGACCGGGTGGCCCGCCCGGGCGTATGTGACGCGTTTGGGGCACCCGGCTGTAGATGCTCT	CGACGTGGGACCCCTTCG	CGACGTGGGACCCCTTCA	ACGTTTTTATGACCCAGACGA
7D_101717316_Stb5_K	Rothamsted Research WAK Promotome Data	*Stb5*	Position 101717316, Chromosome 7D	GGCCCAAACAACCACCTAACATGAAAAATCGAGACATGAAGGACCGCAGGACAGGAGGTGAACCGCGGAACCCTAACCCCGCCCATCGTGTCCTCCCCCCTCTGTTTCAAATCTAATAATCTACAAATACATACGGTGTTACGGTTGCCCTGCCTCCCTTCCCCTCCCCTCCCGTCCTAAGCGTGACCGAAGACAGGCTC[T/A]TCGGCCTGAGCGAGGCTGACAGGGTCATAGTTTTGATTTTTTTCCAAATTATTAGGGCATATATTTTCGGAATTTGTTATAAAAACATTTTACCACGCAACGCAACTGCCCTGACTCGATCTTTCTCCACCCCACTCAGAGCATCTCCACTAGCCTCCCCAGGACACCTTTCTAGGCTTGATTTTAGGCTCGGACGACAC	GAAGGTGACCAAGTTCATGCTCAGCCTCGCTCAGGCCGAA	GAAGGTCGGAGTCAACGGATTCAGCCTCGCTCAGGCCGAT	CCCCTCCCCTCCCGTCCTA

EC_Stb5_7D_97759874_p	Rothamsted Research WAK Promotome Data	*Stb5*	Position 97759874, Chromosome 7D	GCCAGCACGTCCGCGCCCACTGCCATCATCGAGTAGAAGTGGAACATGGGAGGTCACCGCTGTGCTTGGGTCCCATGAAGACCACCACGGAGGCGACGCCGGTGTACATAAGATGTGTCTTGCCTGGTCGCAGGCGACCGTCGTCCCCCGCCCCTCCGGTCGAAGCACATCCCGCAGTGCCACTAAGATGAGCGGTGCTATCAAACATGGGGAAGCGAGCTGCTCTTTGTG[C/T]TGAAGCATATACCTCCGGGGCTCAAGGCAGTCCAATAGCGGGCTGCCAGACATGGGGGAAGACAATGAAGATCCGCCGTGGTTGGCCATAGACTAGTCAAGGGTATCCGTGTCATGGGAATGGATATCCGCCGGTGTCGGTCGTAGACTAGTTTTATCTTAGTCCGGTATATTTTTAGTTAAAAATGCCCAATATGTAATGAATTCATCTGGTTTATATGTAATTCGTCGTGTTTGCATGAA	GCGAGCTGCTCTTTGTGC	GCGAGCTGCTCTTTGTGT	TATTGGACTGCCTTGAGCCC
AX-94418300_k (88191346)	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb5*	Position 88191346, Chromosome 7D	GTTGTATGCGCGTTATGAAAAGAACAAATTTTAACAGTTGTGTAAGCGGG[Y]GCGGTTGTCTCTGTAATTTGAACCCTTGAGCAATGTGGATTTTGCTGCCC	GAAGGTGACCAAGTTCATGCTAAATTTTAACAGTTGTGTAAGCGGGC	GAAGGTCGGAGTCAACGGATTCAAATTTTAACAGTTGTGTAAGCGGGT	TTGCTCAAGGGTTCAAATTACAGAGACAA
7D_89597474_Stb5_K	Rothamsted Research WAK Promotome Data	*Stb5*	Position 89597474, Chromosome 7D	CCGGTCTTCCTTTCTTCGTTGATCTGCCGGTGTCGGCATTTATTTCTCTTTCTTCTTATTGTTTTTCTTTTGGGCTTATCTGTGATGCGGCCCCAGCGAGCTGGATGTATCGGGTGTTTGCTTTATAATATAAAGCGGGGGGAAACCCTTTATCGGAATCAGGATCCCGGGACCAGACACTAGTTTGATCATTGACCAGG[C/G]TCACTAATTTGCAAGAAAAAACAACTTTGTGTAGAATGGTAAAAGGAAATGGACCCTGATACTGAACGTGAGGGTAAAAAAATACCGAACGTCGGGTACGAGCACATGACAACTCATAACGTTTTCGACGGCAAGTTTAATGACGCGAGGATGACAAGTTTAGTTGCCAAGCATGACAACTTTCGTATTTCAGTTTACTT	GAAGGTGACCAAGTTCATGCTACACTAGTTTGATCATTGACCAGGC	GAAGGTCGGAGTCAACGGATTACACTAGTTTGATCATTGACCAGGG	GGTCCATTTCCTTTTACCATTCTACACAA

7D_20495947_ Stb5_K	Rothamsted Research WAK Promotome Data	*Stb5*	Position 20495947, Chromosome 7D	GCCAAAGCTACCACAACACCATGAGCACCCACCACCCCCTCCTCTGGCTACCTGTTGAATATATTGTGCATGTATATTGTGTTGTAGGTCCACCTTCTAGTTTTCCTGTATAGTTGACGTTATGGCTCCGTCTTGTACATCTTATATACGTGTCTAGTGCACCCGATCAATGCATTGTGTGTTGCACGGCCTAAACCTAGTCTTCCACA[G/T]GGTATCAGTTTCCCCCACGATCTTACCCTAGCCTAGCCGTCGCCGCGCCTCCTCTCGCGCCGCCGCCACCCGCCGCTGCTGCCACTCCCTCCAGCCGCGCGTTGCCGCCGCCACCCTTCCTCTTCCGCTTTCGCCACCCCTCTCCTACCTGCAACCCTAGCAGCCGCACGCCCCGACCCGCCCCTCCTTCCCAGCCGCCG	GAAGGTGACCAAGTTCATGCTGGCCTAAACCTAGTCTTCCACAG	GAAGGTCGGAGTCAACGGATTGGCCTAAACCTAGTCTTCCACAT	GGTAAGATCGTGGGGGAAACTGATA
AX-94918531_k	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb7*	Position 719178878, Chromosome 4A	AAGGGTGGATCTGGAAACTTTGTCAAGATGGTTCACAATGGTATTGAGTA[Y]GGTGACATGCAGCTGATCGCTGAGGCGTATGACGTTCTCAAGTCGGTTGG	GAAGGTGACCAAGTTCATGCTGCGATCAGCTGCATGTCACCG	GAAGGTCGGAGTCAACGGATTAGCGATCAGCTGCATGTCACCA	GTCAAGATGGTTCACAATGGTATTGAGTA
AX-94475129_k	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb7*	Position 731006478, Chromosome 4A	CTTATCCAGACTCCCTTTAGGTAGATATTCAAAGCAGAGTAACCTTTTTC[K]CACATCTGCTAAGACAAGCTTTCCCTCACAGTCTACCATTTCCCCTTGTG	GAAGGTGACCAAGTTCATGCTGAAAGCTTGTCTTAGCAGATGTGC	GAAGGTCGGAGTCAACGGATTGGAAAGCTTGTCTTAGCAGATGTGA	CCCTTTAGGTAGATATTCAAAGCAGAGTA
AX-94980296_k	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb7*	Position 743868205, Chromosome 4A	GTCTCTTTAATAACTGCCCTGAGGTATTGCATGCTCTTTAGTGCGGCCTC[K]TGTACTGTAGCCTGTCCTGCGAGAGCACAATCTGCCTCGGACTGCACGCG	GAAGGTGACCAAGTTCATGCTCTTTAGTGCGGCCTCG	GAAGGTCGGAGTCAACGGATTCATGCTCTTTAGTGCGGCCTCT	CTCTCGCAGGACAGGCTACAGTA
AX-95175098	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb7*	Position 740626510, Chromosome 4A	TAGTGGCGTCACATGGTGGTGTCGTCTGATGCAGATCAACTACCAGATGC[Y]ACTTGTGAGGATGATGCGGTGGTGCCAGAGAATCTCAGGATTTTGGTGGT	GAAGGTGACCAAGTTCATGCTACCGCATCATCCTCACAAGTG	GAAGGTCGGAGTCAACGGATTACCGCATCATCCTCACAAGTA	TCGTCTGATGCAGATCAACTAC
AX-94780124_k	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb7*	Position 733433961, Chromosome 4A	TGATTTCCACCGCAAGTTTCTGCATAGGTACATGAAATGATGCTGCTGTA[Y]GCTTTATGTTGTCTGGACAGTATGCATGCTGAAAGAGCCTGTGATGTTTC	GAAGGTGACCAAGTTCATGCTGGTACATGAAATGATGCTGCTGTAC	GAAGGTCGGAGTCAACGGATTAGGTACATGAAATGATGCTGCTGTAT	CAGCATGCATACTGTCCAGACAACAT

AX-94444583_k	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb10*	Position 34619199, Chromosome 1D	ATGCAAAGAAGGCCGAGTCAGAAGTCCATCAGCAGTTTGAGGAATGGAAG[R]ACAAGGCTGGATCACTTGAAATTGAATTGGAGGAGGCTACTCTCTCCGAG	GAAGGTGACCAAGTTCATGCTTCAATTTCAAGTGATCCAGCCTTGTT	GAAGGTCGGAGTCAACGGATTCAATTTCAAGTGATCCAGCCTTGTC	GTCCATCAGCAGTTTGAGGAATGGAA
AX-94798786_k	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb10*	Position 34619771, Chromosome 1D	CTCACTCAGATTGGCGATCTCAAGATGTCCCTAAATAGTACCAAAGAGAA[Y]TATGAGGTAATGATAGAGGAGGCACACTATGACATCACTTGTTTAAGGAA	GAAGGTGACCAAGTTCATGCTATGTCCCTAAATAGTACCAAAGAGAAC	GAAGGTCGGAGTCAACGGATTGATGTCCCTAAATAGTACCAAAGAGAAT	GTGTGCCTCCTCTATCATTACCTCAT
AX-94640607_k	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb11*	Position 57437751, Chromosome 1B	GAGTTGAGGCCGACGCCAGATAGCTTTGCAAACCTGAGCACGAGTTGTTT[M]AGCTTCGGCATGGACCTCGAGATCGCTATTGCTTCCCTGTCGCCGTCCTG	GAAGGTGACCAAGTTCATGCTGCAAACCTGAGCACGAGTTGTTTA	GAAGGTCGGAGTCAACGGATTCAAACCTGAGCACGAGTTGTTTC	CGATCTCGAGGTCCATGCCGAA
AX-94645442_k	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb12*	Position 733706257, Chromosome 4A	CCCCTGGGAAGGTCCGACGGGTCCGGGGGTTACACTTACTCGTCGCGTGG[Y]GGGAGCTCCGGCGGAAGGTGGGACACTCGCTCGACGGGGTCGAGTGACCG	GAAGGTGACCAAGTTCATGCTTACACTTACTCGTCGCGTGGC	GAAGGTCGGAGTCAACGGATTGTTACACTTACTCGTCGCGTGGT	GTCGAGCGAGTGTCCCACCTT
AX-94531033_k	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb12*	Position 735006493, Chromosome 4A	CAGAAGAGGTCCTCCAACATTTGACAGAACTTTCCAGCTTGTCCATCTAT[R]ATTGCTGGTGTCTCAGGTCGTTAAGGGGCTTACGAGCTGCTACCTCTCTT	GAAGGTGACCAAGTTCATGCTTAACGACCTGAGACACCAGCAATT	GAAGGTCGGAGTCAACGGATTACGACCTGAGACACCAGCAATC	ATTTGACAGAACTTTCCAGCTTGTCCAT
AX-94863246_k	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb12*	Position 732512417, Chromosome 4A	GGCAGGACTGGCCTGTTGAACAACAAGCTGTACGTTGTCGGCGGTGTCAT[Y]AGGGAAGAAACTGGATTGGCTCCGCTTCAATCTGCTGAAGTGTTTGACCC	GAAGGTGACCAAGTTCATGCTACGTTGTCGGCGGTGTCATC	GAAGGTCGGAGTCAACGGATTGTACGTTGTCGGCGGTGTCATT	TGAAGCGGAGCCAATCCAGTTTCTT
AX-94893368_k	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb13*	Position 540062500, Chromosome 7B	GTTTGTCAATGTAAAAGAAATAATGTACTGCCCAT[C/T]TTGTCTATTAATTTTGATCGGCTTCCCCTTGTGTT	GAAGGTGACCAAGTTCATGCTAATGTAAAAGAAATAATGTACTGCCCATC	GAAGGTCGGAGTCAACGGATTCAATGTAAAAGAAATAATGTACTGCCCATT	AGGGGAAGCCGATCAAAATTAATAGACAA
AX-94395385_k	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb13*	Position 539786477, Chromosome 7B	GGGAAGCATTTGTCCTCTGGTAGCAGGATGGAGTT[C/T]GTAACTATAACTTCCTCAAAGATGCCCCCTGAAAG	GAAGGTGACCAAGTTCATGCTCTGGTAGCAGGATGGAGTTC	GAAGGTCGGAGTCAACGGATTCCTCTGGTAGCAGGATGGAGTTT	CAGGGGGCATCTTTGAGGAAGTTAT
AX-95202159_k	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb13*	Position 548165329, Chromosome 7B	CCTATCTGCAGAAACCTAATGTGAGGCACCGCCCC[A/T]ACTCGCGGTTCCTTGCCGCCACCGTTCTTGGCGTT	GAAGGTGACCAAGTTCATGCTGCGGCAAGGAACCGCGAGTT	GAAGGTCGGAGTCAACGGATTGCGGCAAGGAACCGCGAGTA	GCACGCCTATCTGCAGAAACCTAAT

AX-94791594_k	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb14*	Position 23600083, Chromosome 3B	CAAGCCATATCGCACGTGATTGCTCTGTTCCTTCT[C/G]CCTATGCACTTCAGGACGCACATTGCTCTGTTATT	GAAGGTGACCAAGTTCATGCTGCGTCCTGAAGTGCATAGGG	GAAGGTCGGAGTCAACGGATTGCGTCCTGAAGTGCATAGGC	ATCGCACGTGATTGCTCTGTTCCTT
AX-94591165_k	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb14*	Position 23364024, Chromosome 3B	CATATTTTGGGGCCAGCAGGGAGAAATCCTCACAG[A/G]AAAGCAAGTACTGATTTTAGTAGCAGAAGCCAACA	GAAGGTGACCAAGTTCATGCTCAGCAGGGAGAAATCCTCACAGA	GAAGGTCGGAGTCAACGGATTAGCAGGGAGAAATCCTCACAGG	GCTTCTGCTACTAAAATCAGTACTTGCTT
AX-94591165_k	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb14*	Position 23364024, Chromosome 3B	CATATTTTGGGGCCAGCAGGGAGAAATCCTCACAG[A/G]AAAGCAAGTACTGATTTTAGTAGCAGAAGCCAACA	GAAGGTGACCAAGTTCATGCTCAGCAGGGAGAAATCCTCACAGA	GAAGGTCGGAGTCAACGGATTAGCAGGGAGAAATCCTCACAGG	CCTGCTGTTGGCTTCTGCTACTAAA
AX-94790051_k	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb14*	Position 821089277, Chromosome 3B	CTGTCTGTCTTCGGAAGTACTCCTGAAATGATGTG[C/T]TGTTACCTTCTTACTCTACTGCTTTCGTCTTTTGC	GAAGGTGACCAAGTTCATGCTGGAAGTACTCCTGAAATGATGTGC	GAAGGTCGGAGTCAACGGATTCGGAAGTACTCCTGAAATGATGTGT	AAGACGAAAGCAGTAGAGTAAGAAGGTAA
AX-94790051_k	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb14*	Position 821089277, Chromosome 3B	CTGTCTGTCTTCGGAAGTACTCCTGAAATGATGTG[C/T]TGTTACCTTCTTACTCTACTGCTTTCGTCTTTTGC	GAAGGTGACCAAGTTCATGCTGGAAGTACTCCTGAAATGATGTGC	GAAGGTCGGAGTCAACGGATTCGGAAGTACTCCTGAAATGATGTGT	GCCAAGGCAAAAGACGAAAGCAGTA
AX-94937231_k	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb17*	Position 531239743, Chromosome 5A	ACTGCTACTTGCCACGCATGCTCGGTGCATAGCGTCCTTGATATTGCAGC[M]GGACTAGCAGTCGGACTAGCTGCGGGACTCACCGGCGGATGGTGCAGCTG	GAAGGTGACCAAGTTCATGCTAGCTAGTCCGACTGCTAGTCCT	GAAGGTCGGAGTCAACGGATTGCTAGTCCGACTGCTAGTCCG	CTCGGTGCATAGCGTCCTTGATATT
AX-94447473_k	RAGT Seeds Ltd. 35K Axiom Breeders Array data	*Stb17*	Position 529808043, Chromosome 5A	TGATTTAGACAACAAGGGTGCCATTCCTCCTCACTGCGCCACATCGGCGTGCTCGATGTTGACGATG[R]GGGTGCCGTCTCTCCACCTCGTTGTGCCACACC	GAAGGTGACCAAGTTCATGCTGCGTGCTCGATGTTGACGATGA	GAAGGTCGGAGTCAACGGATTCGTGCTCGATGTTGACGATGG	ACAACGAGGTGGAGAGACGGCA

EC_Stb18_6D_21076387_p	Rothamsted Research WAK Promotome Data	*Stb18*	Position 21076387, Chromosome 6D	GTGGAAGAGCCAGGTTTGTTTCGCATGCTGTTGACCCACACGATTAAGATTCGAGGTTGATCTCACAGATTCAGTCTAATCATGGCACACACAACTCATACATGTGTGCTAGGGACTAGTTTATTTAGACTCAAAAAACTGGAATGTTTTATCATGATTGATAACATTGCCCATGAGAGACACGG[A/G]TTTTTCCAAAAATAGGTGCAATAATAAAACAACTGAAAATAGAGTCTATAAATAAGTCCCTATACTCTAGCCTCAATATGCATTATGAGCAGCATCAAGGAAAAGACAGAGAAATAAGAGGGTAGACTGCATAGCCCATAATTCCCAACGAGGCAGTCTTAGTGAGGGAGAAAAGAGGCTATGAACCCGAAAAAAAAA	GAAGGTGACCAAGTTCATGCTTTGCCCATGAGAGACACGGA	GAAGGTCGGAGTCAACGGATTTTGCCCATGAGAGACACGGG	CCTTGATGCTGCTCATAATGCA
EC_Stb18_6D_21076387_k	Rothamsted Research WAK Promotome Data	*Stb18*	Position 21076387, Chromosome 6D	GTGGAAGAGCCAGGTTTGTTTCGCATGCTGTTGACCCACACGATTAAGATTCGAGGTTGATCTCACAGATTCAGTCTAATCATGGCACACACAACTCATACATGTGTGCTAGGGACTAGTTTATTTAGACTCAAAAAACTGGAATGTTTTATCATGATTGATAACATTGCCCATGAGAGACACGG[A/G]TTTTTCCAAAAATAGGTGCAATAATAAAACAACTGAAAATAGAGTCTATAAATAAGTCCCTATACTCTAGCCTCAATATGCATTATGAGCAGCATCAAGGAAAAGACAGAGAAATAAGAGGGTAGACTGCATAGCCCATAATTCCCAACGAGGCAGTCTTAGTGAGGGAGAAAAGAGGCTATGAACCCGAAAAAAAAA	GAAGGTGACCAAGTTCATGCTCATTGCCCATGAGAGACACGGA	GAAGGTCGGAGTCAACGGATTGCCCATGAGAGACACGGG	GTTGTTTTATTATTGCACCTATTTTTGGAA
EC_Stb18_6D_25537253_k	Rothamsted Research WAK Promotome Data	*Stb18*	Position 25537253, Chromosome 6D	TGATTTGAATGAGTTAATGCATGACATTATTTTGGGAAAGTGCCGATTTAATTGAGTTAATGCATGTGTCAAATGGGCCGGTCTAAATTGGCTGCACTTGAGCGAAATATCATCCGAAGACGCTTACGGGCAAAATATAGTGTTTGCCTACGAATTAATGAGAGTGAGGCAAGACTGCCAACTCC[C/T]CATTTAGAAGTAGAGATTTAGATGGTGTCATCCCAACATGTCGATTTTAGTGATCCTATACGTAAAGACTTGAACAAATGTAATTTGCCCCATGGAGGCATTATAATTTGAAACAGTCAAAGTTTTGACATAGATTCCAATGATTTTTGTTCTTTTCAAAATTATCTTCTTCTATTAGACTATTACCAACAAATATAT	GAAGGTGACCAAGTTCATGCTGAGGCAAGACTGCCAACTCCC	GAAGGTCGGAGTCAACGGATTGAGGCAAGACTGCCAACTCCT	CGACATGTTGGGATGACACCATCTA

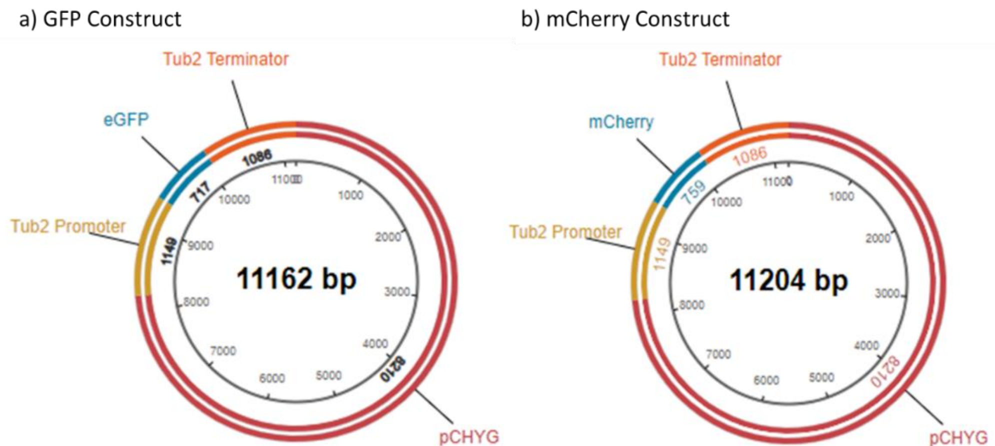
EC_Stb18_6D_25537253_p	Rothamsted Research WAK Promotome Data	*Stb18*	Position 25537253, Chromosome 6D	TGATTTGAATGAGTTAATGCATGACATTATTTTGGGAAAGTGCCGATTTAATTGAGTTAATGCATGTGTCAAATGGGCCGGTCTAAATTGGCTGCACTTGAGCGAAATATCATCCGAAGACGCTTACGGGCAAAATATAGTGTTTGCCTACGAATTAATGAGAGTGAGGCAAGACTGCCAACTCC[C/T]CATTTAGAAGTAGAGATTTAGATGGTGTCATCCCAACATGTCGATTTTAGTGATCCTATACGTAAAGACTTGAACAAATGTAATTTGCCCCATGGAGGCATTATAATTTGAAACAGTCAAAGTTTTGACATAGATTCCAATGATTTTTGTTCTTTTCAAAATTATCTTCTTCTATTAGACTATTACCAACAAATATAT	GCAAGACTGCCAACTCCC	GCAAGACTGCCAACTCCT	CGACATGTTGGGATGACACC
EC_Stb18_6D_24377769_p	Rothamsted Research WAK Promotome Data	*Stb18*	Position 24377769, Chromosome 6D	AGCTGGTTGGGCAGCTCGCCGCCGGCGGCCCAGCACCTCCTGTGACCTGAGCAGCCGGCGCCGAGATGCAGAGCTGGAACAGCAGCACAATGAACGCGGCCGCCGGAATCATCGTGATGATGCGTGTAAAACCCTCGTCGTACCG[A/T]CGCGGGATATGCAGCTGCGTGCGACGAATATATGCTATCGGTCCATGCATGCAGTGAGTCAGCCCATGCATTGATATTCACGCGTGGACTGACCATGTCTACGCCGACTAGTATATATCACTGTTCATTTTCCTTGCAACAGAAGCAATA	AAAACCCTCGTCGTACCGA	AAAACCCTCGTCGTACCGT	CATGGGCTGACTCACTGCA
EC_Stb18_6D_24387369_k	Rothamsted Research WAK Promotome Data	*Stb18*	Position 24387369, Chromosome 6D	CCGCCGCCGGTCACGTGCAGCTGCGTGAGAGGCATATATGCTGTCGGTCGGTCTATGCATGCAGTGAGTCAGCGCAT[G/C]TGTTCATATTCACGCGTGGACTGACCTTGTCTATATCACTGTTCATTTTGCTTCCAACAGAAGCAATAATAGCACTGTACGTACGCGCGG	GAAGGTGACCAAGTTCATGCTCATGCAGTGAGTCAGCGCATG	GAAGGTCGGAGTCAACGGATTCATGCAGTGAGTCAGCGCATC	AGGTCAGTCCACGCGTGAATATGAA
EC_Stb18_6D_24377838_k	Rothamsted Research WAK Promotome Data	*Stb18*	Position 24377838, Chromosome 6D	AGAGCTGGAACAGCAGCACAATGAACGCGGCCGCCGGAATCATCGTGATGATGCGTGTAAAACCCTCGTCGTACCGACGCGGGATATGCAGC[T/A]AGCGTGCGACGAATATATGCTATCGGTCCATGCATGCAGTGAGTCAGCCCATGCATTGATATTCACGCGTGGACTGACCATGTCTACGCCGACTAGTATA	GAAGGTGACCAAGTTCATGCTGATAGCATATATTCGTCGCACGCTA	GAAGGTCGGAGTCAACGGATTATAGCATATATTCGTCGCACGCTG	TGATGCGTGTAAAACCCTCGTCGTA

EC_Stb18_6D_ 24387417_k	Rothamsted Research WAK Promotome Data	*Stb18*	Position 24387417, Chromosome 6D	TCAGCGCATGTGTTCATATTCACGCG TGGACTGACCTTGTCTATATCACTGT TCATTTTGCTTCCAACAGAAGCAATA ATAGCACTGTAC[G/A]TACGCGCGGA CCTTTTCAAGGCCGTGATTATTAGTA TTATTTTTTATTATCTTACTAAAGGAT GGAGCAAAAATCTGAATTTTGGACCG ACAACGTCTTTA	GAAGGTGAC CAAGTTCAT GCTAACAGA AGCAATAAT AGCACTGTA CG	GAAGGTCGG AGTCAACGG ATTCAACAG AAGCAATAA TAGCACTGT ACA	GCCTTGAA AAGGTCCG CGCGTA
EC_Stb18_6D_ 24377769_k	Rothamsted Research WAK Promotome Data	*Stb18*	Position 24377769, Chromosome 6D	AGCTGGTTGGGCAGCTCGCCGCCGG CGGCCCAGCACCTCCTGTGACCTGA GCAGCCGGCGCCGAGATGCAGAGCT GGAACAGCAGCACAATGAACGCGGC CGCCGGAATCATCGTGATGATGCGT GTAAAACCCTCGTCGTACCG[A/T]CG CGGGATATGCAGCTGCGTGCGACGA ATATATGCTATCGGTCCATGCATGCA GTGAGTCAGCCCATGCATTGATATTC ACGCGTGGACTGACCATGTCTACGC CGACTAGTATATATCACTGTTCATTTT CCTTGCAACAGAAGCAATA	GAAGGTGAC CAAGTTCAT GCTGTGTAA AACCCTCGT CGTACCGA	GAAGGTCGG AGTCAACGG ATTGTGTAA AACCCTCGT CGTACCGT	CTGCATGC ATGGACCG ATAGCATA T
EC_Stb18_6D_ 24379384_p	Rothamsted Research WAK Promotome Data	*Stb18*	Position 24379384, Chromosome 6D	CCCATACCCTTATATACCACTTAAATT TCCCTTTCCCAAATATGTCATGGAAA TTTTGCATCGCAATATTCCACTACTG CCAGTTGACCGTTACTTGACAATTGA AAAAGACAAAAATTCCCTTG[A/G]GCC ATACCTTGTGACCATTTAGCTAAATG ATCTACTGATTTTGGTGTACTCCATC ACCAGGTTGCAGCTCAAGAGGAAAA CAAGGAATTGAGAGAGAACCAGAGA GATACAGGCCACCGGGCTAGGGCG GA	AGCTAAATG GTCACAAGG TATGGT	AGCTAAATG GTCACAAGG TATGGA	ACTGCCAG TTGACCGT TACT
EC_Stb18_6D_ 24378137_k	Rothamsted Research WAK Promotome Data	*Stb18*	Position 24378137, Chromosome 6D	AAAATACTTAAAATTATAAGTGGGTCT TTGTCCCAATTTACATTAACGAGACA TAGGGTAGAGTGTATTTTTAGGTATA TCATGGTAAAATCTAAAAATGTGCCA TTATGTCCTTAGAAA[G/A]GAATACAC TCTCCATGTCAGTATATCTCTTTGAC GTTAATGTAAGAAATAGGAGTAAAGA AAACCTCAAACAATACTCGCTTTGTTT CAGGGTGATTGATCGACCTTTTAGAT TATTGTTGGTTGATCACTTTGT	GAAGGTGAC CAAGTTCAT GCTAAAAAT GTGCCATTA TGTCCTTAG AAAG	GAAGGTCGG AGTCAACGG ATTCTAAAAA TGTGCCATT ATGTCCTTA GAAAA	TACATTAA CGTCAAAG AGATATAC TGACAT

EC_Stb18_6D_24378137_p	Rothamsted Research WAK Promotome Data	*Stb18*	Position 24378137, Chromosome 6D	AAAATACTTAAAATTATAAGTGGGTCTTTGTCCCAATTTACATTAACGAGACATAGGGTAGAGTGTATTTTTAGGTATATCATGGTAAAATCTAAAAATGTGCCATTATGTCCTTAGAAA[G/A]GAATACACTCTCCATGTCAGTATATCTCTTTGACGTTAATGTAAGAAATAGGAGTAAAGAAAACCTCAAACAATACTCGCTTTGTTTCAGGGTGATTGATCGACCTTTTAGATTATTGTTGGTTGATCACTTTGT	AATGTGCCATTATGTCCTTAGAAAG	AATGTGCCATTATGTCCTTAGAAAA	CACCCTGAAACAAAGCGAGT
EC_Stb18_6D_24387417_p	Rothamsted Research WAK Promotome Data	*Stb18*	Position 24387417, Chromosome 6D	TCAGCGCATGTGTTCATATTCACGCGTGGACTGACCTTGTCTATATCACTGTTCATTTTGCTTCCAACAGAAGCAATAATAGCACTGTAC[G/A]TACGCGCGGACCTTTTCAAGGCCGTGATTATTAGTATTATTTTTTATTATCTTACTAAAGGATGGAGCAAAAATCTGAATTTTGGACCGACAACGTCTTTA	TTGAAAAGGTCCGCGCGTAC	TTGAAAAGGTCCGCGCGTAT	TATTCACGCGTGGACTGACC
EC_Stb18_6D_25727651_k	Rothamsted Research WAK Promotome Data	*Stb18*	Position 25727651, Chromosome 6D	CAGGATTGGATTGGATTAAGCGGAAAGAAACCATCAAAGGCGGCACAGCAAGGCGTGAGTGGATAACAGAACGGGAACGTTTGCAACGCACGCACGCACTCACGATCGATGCCAGCCAGCCTCCATCTATCACCGCCAGCCTGTCTCTCTACATCTCTCTCTTGGCGTACGTCAGGTATGGCATA[T/C]ATATGGACTTCATTCAGGTGGACGTCAGAACTCTGAAAGCTCCCTCCCTCGGATCATCGAGATCTCACCGCTTCATTCCTTGTCGCTGCTCCGCCTTCGCCTTCTTCTTCTTCCTCTCGCCCGCGTCTTCCGTCGTACCATGCAGAGCTTCTTCGGTCGAGGCAGCAGGTAAAGCCCAACAGATTAAATTCCTTTTTG	GAAGGTGACCAAGTTCATGCTGGCGTACGTCAGGTATGGCATAT	GAAGGTCGGAGTCAACGGATTGCGTACGTCAGGTATGGCATAC	GACGTCCACCTGAATGAAGTCCATA
EC_Stb18_6D_26241560_k	Rothamsted Research WAK Promotome Data	*Stb18*	Position 26241560, Chromosome 6D	TATATTGTCTTGTGACATTCAAGTTCAATTATTTATTGTTGAAAAATAAAAATAAAAATAATGGTGTGGGGGCCTTTCATGTTCTTTGAT[G/T]ATGAGAGGGCTTGCATGTGGTAGAAAGTGAAAAGTTAAATGATTGCATGGGCACTTGATGATGTGGAGTGCGTGCAAGTGTTGAAAAGTAAAAGGTTGATT	GAAGGTGACCAAGTTCATGCTACCACATGCAAGCCCTCTCATC	GAAGGTCGGAGTCAACGGATTACCACATGCAAGCCCTCTCATA	GTGGGGGCCTTTCATGTTCTTTGAT

EC_Stb18_6D_ 26241560_p	Rothamsted Research WAK Promotome Data	*Stb18*	Position 26241560, Chromosome 6D	TATATTGTCTTGTGACATTCAAGTTCAATTATTTATTGTTGAAAAAATAAAAAATAAAAATAATGGTGTGGGGGGCCTTTCATGTTCTTTGAT[G/T]ATGAGAGGGCTTGCATGTGGTAGAAAGTGAAAAGTTAAATGATTGCATGGGCACTTGATGATGTGGAGTGCGTGCAAGTGTTGAAAAGTAAAAGGTTGATT	GGGGCCTTTCATGTTCTTTGATG	GGGGCCTTTCATGTTCTTTGATT	TCCACATCATCAAGTGCCCA

Chapter 4 - The Production and Testing of Fluorescent *Z. tritici* Research Tools for the Examination of *Stb* Resistance Gene Mechanisms – Supplementary Figures



Supplementary Figure 1: The Gibson Assembly constructs containing a) the Green Fluorescent Protein (GFP) fluorophore and b) the mCherry fluorophore, used in *E. coli* and *Agrobacterium*.

GFP Final Assembly Sequence

TTAAGAGGAGTCCACCATGGTAGATCTGACTAGTGTTAACG
CTAGCCACCACCACCACCACCACGTGTGAATTACAGGTGAC
CAGCTCGAATTTCCCCGATCGTTCAAACATTTGGCAATAAA
GTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTA
TCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAA
CATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGAT
TAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAA
AATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGT
CATCTATGTTACTAGATCGGGAATTAAACTATCAGTGTTTGA
CAGGATATATTGGCGGGTAAACCTAAGAGAAAAGAGCGTTT
ATTAGAATAACGGATATTTAAAAGGGCGTGAAAAGGTTTATC
CGTTCGTCCATTTGTATGTGCATGCCAACCACAGGGTTCCC
CTCGGGATCAAAGTACTTTGATCCAACCCCTCCGCTGCTAT
AGTGCAGTCGGCTTCTGACGTTCAGTGCAGCCGTCTTCTGA
AAACGACATGTCGCACAAGTCCTAAGTTACGCGACAGGCTG
CCGCCCTGCCCTTTTCCTGGCGTTTTCTTGTCGCGTGTTTT
AGTCGCATAAAGTAGAATACTTGCGACTAGAACCGGAGACA
TTACGCCATGAACAAGAGCGCCGCCGCTGGCCTGCTGGGC
TATGCCCGCGTCAGCACCGACGACCAGGACTTGACCAACC
AACGGGCCGAACTGCACGCGGCCGGCTGCACCAAGCTGTT
TTCCGAGAAGATCACCGGCACCAGGCGCGACCGCCCGGAG
CTGGCCAGGATGCTTGACCACCTACGCCCTGGCGACGTTG
TGACAGTGACCAGGCTAGACCGCCTGGCCCGCAGCACCCG
CGACCTACTGGACATTGCCGAGCGCATCCAGGAGGCCGGC
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CCACGCCGGCCGGCCGCATGGTGTTGACCGTGTTCGCCGG

CATTGCCGAGTTCGAGCGTTCCCTAATCATCGACCGCACCC
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TTGGCCCCCGCCCTACCCTCACCCCGGCACAGATCGCGCA
CGCCCGCGAGCTGATCGACCAGGAAGGCCGCACCGTGAAA
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GGCCAGGCGGCGCGGTGCCTTCCGTGAGGACGCATTGACC
GAGGCCGACGCCCTGGCGGCCGCCGAGAATGAACGCCAAG
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GCTGGCGGCCTGGCCGGCCAGCTTGGCCGCTGAAGAAACC
GAGCGCCGCCGTCTAAAAAGGTGATGTGTATTTGAGTAAAA
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CATCGGCCGGCGCGACTTCGTAGTGATCGACGGAGCGCCC
CAGGCGGCGGACTTGGCTGTGTCCGCGATCAAGGCAGCCG
ACTTCGTGCTGATTCCGGTGCAGCCAAGCCCTTACGACATA
TGGGCCACCGCCGACCTGGTGGAGCTGGTTAAGCAGCGCA
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TCGCGGGCGATCAAAGGCACGCGCATCGGCGGTGAGGTTG
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CCGTATCACGCAGCGCGTGAGCTACCCAGGCACTGCCGCC
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CAATGGCACTGGAACCCCCAAGCCCGAGGAATCGGCGTGA
CGGTCGCAAACCATCCGGCCCGGTACAAATCGGCGCGGCG
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CTGCCGGCGAGCCGATGGCGGAAAGCAGAAAGACGACCTG
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GCAGCGTACGAAGAAGGCCAAGAACGGCCGCCTGGTGACG
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mCherry Final Assembly Sequence

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C A C G C A C A C A G C C A C C A T C A G T G G T C C C A G T T C A G C T G T C T
C A A T C A T G C G T G A C A T G T C G G C C A C G T C G A C G T G C T G C A A G
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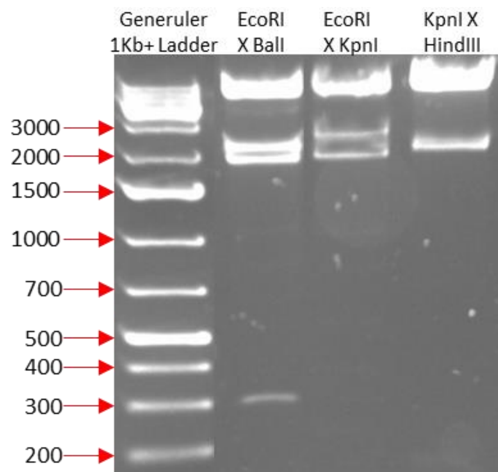
C A T G C G T G A A T A T T G C T A C C T C T C T C A T A A G A G C T T G G C T G
T C G A C T C C T C

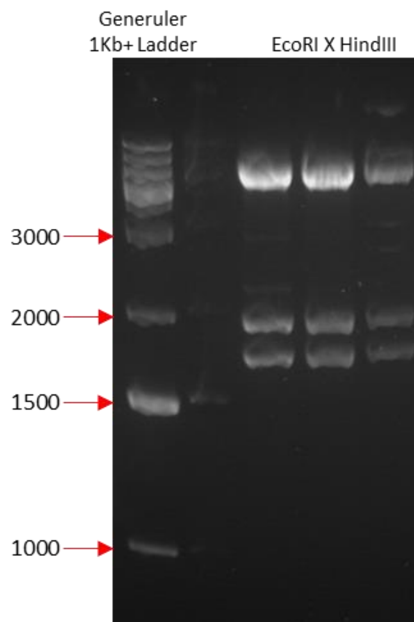
Supplementary Figure 2: The full sequences for the Gibson Assembly constructs containing Green Fluorescent Protein (GFP) fluorophore and mCherry fluorophores.

Tub2-Prom-F: TGACAAGATGGCAGTCGACGCCAGATGATG
Tub2-Prom-R: TGCTCACCATGGCGATGGTGGTATGCGG
eGFP/mCherry-F: CACCATCGCCATGGTGAGCAAGGGCGAG
eGFP-R: CCGTCGTCGCCTTGTACAGCTCGTCCATGC
mCherry-R: CCGTCGTCGCTCATTGCCAAATGTTTGAACGATC
Tub2-Term-F (GFP): GCTGTACAAGGCGACGACGGACGAGGAC
Tub2-Term-F (mCh): TTGGCAATGAGCGACGACGGACGAGGAC
Tub2-Term-R: TTGAATCTGAGAGGAGTCGACAGCCAAGC

Supplementary Figure 3: A list of the oligoes used to produce the final GFP and mCherry constructs.



Supplementary Figure 4a: GFP construct double digest gel showing bands of the correct sizes for three different combinations of restriction enzymes. The band sizes in base pairs for the Generuler 1Kb+ Ladder used are listed beside.



Supplementary Figure 4b: mCherry construct double digest gel showing bands of the correct sizes for three different digests using the restriction enzymes EcoRI and HindIII. The band sizes in base pairs for the Generuler 1Kb+ Ladder used are listed beside.

Supplementary Table 1: This table shows the proportion of open stomata, germinated spores and successful and unsuccessful stomatal penetration attempts on the surfaces of wheat leaves inoculated with fluorescent, wild type and mixed co-inoculations of RResHT-1 and RResHT-52 *Z. tritici* strains and isolates over 12 days post inoculation. Greyed out cells indicate a lack of measurable results.

Wheat genotype	DPI	Measurement made	Mock	RResHT-1-WT	RResHT-52-WT	RResHT-1-mCherry	RResHT-52-GFP	RResHT-1-mCherry and RResHT-52-GFP co-inoculation
KWS Cashel	1	% of stomata that were open	-	-	-	11.1	0.0	0.0
		% of spores that germinated successfully	-	-	-	11.8	0.0	30.0
		% of stomata reached by epiphytic hyphae	-	-	-	22.2	0.0	11.1
		% of cases where epiphytic hyphae reaching stomata led to a penetration attempt	-	-	-	0/2	No stomata reached by hyphae	0/1
		% of penetration attempts that were successful	-	-	-	No penetration attempts made	No penetration attempts made	No penetration attempts made
	3	% of stomata that were open	60.0	25.0	0.0	75.0	71.4	83.3
		% of spores that germinated successfully	No spores visible	0.0	0.0	0.0	14.3	20.0
		% of stomata reached by epiphytic hyphae	0.0	0.0	0.0	0.0	0.0	0.0
		% of cases where epiphytic hyphae reaching stomata led to a penetration attempt	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae
		% of penetration attempts that were successful	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made
	5	% of stomata that were open	89.5	87.5	80.0	52.9	100.0	80.0
		% of spores that germinated successfully	No spores visible	0.0	43.8	28.6	34.4	12.5
		% of stomata reached by epiphytic hyphae	0.0	0.0	10.0	5.9	15.0	0.0
		% of cases where epiphytic hyphae reaching stomata led to a penetration attempt	No stomata reached by hyphae	No stomata reached by hyphae	0/1	0/1	0.3	No stomata reached by hyphae
		% of penetration attempts that were successful	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made	1/1	No penetration attempts made
	8	% of stomata that were open	100.0	75.0	28.6	71.4	28.6	100.0

		% of spores that germinated successfully	No spores visible	50.0	0.0	33.3	43.8	100.0
		% of stomata reached by epiphytic hyphae	0.0	16.7	0.0	0.0	7.1	0.0
		% of cases where epiphytic hyphae reaching stomata led to a penetration attempt	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae
		% of penetration attempts that were successful	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made
	12	% of stomata that were open	0.0	61.5	33.3	68.8	0.0	0.0
		% of spores that germinated successfully	0.0	44.4	72.2	39.1	41.2	33.3
		% of stomata reached by epiphytic hyphae	0.0	7.7	8.3	0.0	0.0	0.0
		% of cases where epiphytic hyphae reaching stomata led to a penetration attempt	No stomata reached by hyphae	1/1	0/1	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae
		% of penetration attempts that were successful	No penetration attempts made	0/1	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made
Synthetic 6X	1	% of stomata that were open	-	-	-	0.0	0.0	10.0
		% of spores that germinated successfully	-	-	-	6.7	16.7	6.7
		% of stomata reached by epiphytic hyphae	-	-	-	16.7	14.3	10.0
		% of cases where epiphytic hyphae reaching stomata led to a penetration attempt	-	-	-	0/1	0/1	0/1
		% of penetration attempts that were successful	-	-	-	No penetration attempts made	No penetration attempts made	No penetration attempts made
	3	% of stomata that were open	46.2	28.6	64.7	0.0	36.4	33.3
		% of spores that germinated successfully	0.0	0.0	23.5	0.0	11.1	0.0
		% of stomata reached by epiphytic hyphae	0.0	0.0	0.0	0.0	0.0	0.0
		% of cases where epiphytic hyphae reaching stomata led to a penetration attempt	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae
		% of penetration attempts that were successful	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made
	5	% of stomata that were open	60.0	100.0	75.0	50.0	100.0	80.0
		% of spores that germinated successfully	0.0	35.7	58.3	12.5	46.8	52.6

		% of stomata reached by epiphytic hyphae	0.0	0.0	0.0	0.0	100.0	0.0
		% of cases where epiphytic hyphae reaching stomata led to a penetration attempt	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae	2/4	No stomata reached by hyphae
		% of penetration attempts that were successful	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made	1/2	No penetration attempts made
	8	% of stomata that were open	0.0	100.0	0.0	0.0	37.5	0.0
		% of spores that germinated successfully	100.0	100.0	50.0	100.0	50.0	50.0
		% of stomata reached by epiphytic hyphae	0.0	0.0	0.0	0.0	12.5	27.3
		% of cases where epiphytic hyphae reaching stomata led to a penetration attempt	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae	1/1	0/3
		% of penetration attempts that were successful	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made	1/1	No penetration attempts made
	12	% of stomata that were open	6.3	28.6	11.1	0.0	0.0	22.2
		% of spores that germinated successfully	100.0	46.2	50.0	25.0	42.3	42.9
		% of stomata reached by epiphytic hyphae	6.3	42.9	11.1	0.0	27.3	11.1
		% of cases where epiphytic hyphae reaching stomata led to a penetration attempt	0/1	2/3	0/1	No stomata reached by hyphae	1/3	0/1
		% of penetration attempts that were successful	No penetration attempts made	2/2	No penetration attempts made	No penetration attempts made	0/1	No penetration attempts made
Chinese Spring NIL -*Stb6*	1	% of stomata that were open	-	-	-	88.2	0.0	0.0
		% of spores that germinated successfully	-	-	-	10.5	14.3	23.1
		% of stomata reached by epiphytic hyphae	-	-	-	11.8	0.0	20.0
		% of cases where epiphytic hyphae reaching stomata led to a penetration attempt	-	-	-	0/2	No stomata reached by hyphae	0
		% of penetration attempts that were successful	-	-	-	No penetration attempts made	No penetration attempts made	No penetration attempts made
	3	% of stomata that were open	53.8	38.9	50.0	28.6	66.7	100.0
		% of spores that germinated successfully	No spores visible	33.3	0.0	9.1	20.6	25.0
		% of stomata reached by epiphytic hyphae	0.0	0.0	0.0	9.1	0.0	0.0

		% of cases where epiphytic hyphae reaching stomata led to a penetration attempt	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae	0/2	No stomata reached by hyphae	No stomata reached by hyphae
		% of penetration attempts that were successful	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made
	5	% of stomata that were open	88.9	100.0	100.0	50.0	75.0	50.0
		% of spores that germinated successfully	75.0	60.0	28.6	29.7	47.4	0.0
		% of stomata reached by epiphytic hyphae	0.0	0.0	33.3	25.0	50.0	0.0
		% of cases where epiphytic hyphae reaching stomata led to a penetration attempt	No stomata reached by hyphae	No stomata reached by hyphae	1/1	0/1	1/4	No stomata reached by hyphae
		% of penetration attempts that were successful	No penetration attempts made	No penetration attempts made	1/1	No penetration attempts made	0/1	No penetration attempts made
	8	% of stomata that were open	-	16.7	50.0	33.3	37.5	12.5
		% of spores that germinated successfully	-	50.0	46.7	50.0	100.0	50.0
		% of stomata reached by epiphytic hyphae	-	0.0	0.0	0.0	0.0	0.0
		% of cases where epiphytic hyphae reaching stomata led to a penetration attempt	-	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae
		% of penetration attempts that were successful	-	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made
	12	% of stomata that were open	5.9	15.4	42.9	9.1	33.3	50.0
		% of spores that germinated successfully	No spores visible	40.0	0.0	44.4	60.0	40.0
		% of stomata reached by epiphytic hyphae	0.0	0.0	0.0	9.1	0.0	16.7
		% of cases where epiphytic hyphae reaching stomata led to a penetration attempt	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae	1/1	No stomata reached by hyphae	0/2
		% of penetration attempts that were successful	No penetration attempts made	No penetration attempts made	No penetration attempts made	1/1	No penetration attempts made	No penetration attempts made
Chinese Spring NIL +*Stb5*	1	% of stomata that were open	20.0	30.0	33.3	42.9	9.1	0.0
		% of spores that germinated successfully	No spores visible	6.7	20.4	11.9	27.3	11.8
		% of stomata reached by epiphytic hyphae	0.0	10.0	11.1	0.0	0.0	0.0

		% of cases where epiphytic hyphae reaching stomata led to a penetration attempt	No stomata reached by hyphae	0/1	0/1	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae
		% of penetration attempts that were successful	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made
	3	% of stomata that were open	75.0	100.0	0.0	22.2	50.0	83.3
		% of spores that germinated successfully	No spores visible	16.7	0.0	16.7	25.6	33.3
		% of stomata reached by epiphytic hyphae	0.0	0.0	20.0	11.1	25.0	0.0
		% of cases where epiphytic hyphae reaching stomata led to a penetration attempt	No stomata reached by hyphae	No stomata reached by hyphae	0/1	0/1	0/1	No stomata reached by hyphae
		% of penetration attempts that were successful	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made
	5	% of stomata that were open	10.0	100.0	40.0	42.9	12.5	66.7
		% of spores that germinated successfully	No spores visible	50.0	47.4	26.4	42.5	40.0
		% of stomata reached by epiphytic hyphae	0.0	66.7	0.0	14.3	50.0	0.0
		% of cases where epiphytic hyphae reaching stomata led to a penetration attempt	No stomata reached by hyphae	0/2	No stomata reached by hyphae	0/1	0/4	No stomata reached by hyphae
		% of penetration attempts that were successful	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made
	8	% of stomata that were open	47.1	28.6	60.0	20.0	17.6	0.0
		% of spores that germinated successfully	0.0	30.0	75.0	48.5	75.0	28.6
		% of stomata reached by epiphytic hyphae	0.0	0.0	0.0	0.0	5.9	0.0
		% of cases where epiphytic hyphae reaching stomata led to a penetration attempt	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae	No stomata reached by hyphae	0/1	No stomata reached by hyphae
		% of penetration attempts that were successful	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made	No penetration attempts made
	12	% of stomata that were open	0.0	50.0	46.2	33.3	18.2	0.0
		% of spores that germinated successfully	No spores visible	41.4	75.0	40.0	52.5	45.5
		% of stomata reached by epiphytic hyphae	0.0	9.1	0.0	16.7	72.7	0.0

		% of cases where epiphytic hyphae reaching stomata led to a penetration attempt	No stomata reached by hyphae	0/2	No stomata reached by hyphae	1/1	3/8	No stomata reached by hyphae
		% of penetration attempts that were successful	No penetration attempts made	No penetration attempts made	No penetration attempts made	0/1	0/3	No penetration attempts made