Disease escape in relation to a trade off between septoria tritici blotch and yield of wheat.

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Abstract

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Zymoseptoria tritici, the fungus that causes Septoria tritici blotch (STB) of wheat, is spread by splash borne transfer from the base of the plant to the flag leaf. This project is on a potential new source of resistance to STB discovered on chromosome 6A using association mapping (Arraiano & Brown 2016). Near isogenic lines generated for this region show no significant differences in STB symptoms when leaves are directly inoculated with *Z. tritici*. However, trials that are naturally infected or inoculated at the base of the plant show clear differences in their level of STB. This indicated that this region contains genes that cause differences in disease escape. The fact that the same marker Psp3071 is associated with yield traits (Snape et al, 2007) led to the hypothesis that the region may control a physiological trait that improves yield at the cost of aiding spore transmission. Candidate physiological traits, that could influence disease escape, have been tested in the 6A NILs including plant height, leaf area and senescence. The trait that fits with the pattern of the disease results best is leaf emergence, with later emerging leaves getting more STB. However, the effect of the 6A alleles on disease escape may be caused by multiple traits. Recombinant lines generated for the region have reduced the interval that contains the yield traits, though it is still unclear if the disease escape and yield effects are connected by linkage or pleiotropy.

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1 General Introduction

1.1 Improving wheat yield

Wheat covers more area on the planet than any other crop, with only Rice and Maize being produced at similar levels. In 2013-2014 wheat was grown on 221,166,000 hectares worldwide (Agrimoney.com, 2015). Increases in the population of the earth have been largely met with increases in crop productivity so far, however many predictions indicate that demand is rising faster than current rates of improvement (Rosegrant and Cline, 2003, Godfray et al., 2010). Climate change may also cause problems for the current varieties grown, increasing the need for crop improvement.

There are many ways that the yield of wheat has been improved over the history of wheat breeding. The yield of wheat is affected by the amount of light intercepted by the plant, the radiation use efficiency and the plants harvest index. Selection for yield has led to increases in the harvest index (Sayre et al., 1995). However, there is a theoretical maximum for HI of about 0.6, and many current varieties are approaching this figure (Austin et al., 1980) . Modern research into yield improvement is increasing focused on improving radiation use efficiency. For example work is being performed on, adopting the more efficient C4 system in C3 plants, increasing the concentration of CO_2 around Rubisco, reducing photorespiration and modifying the inhibitors of Rubisco (Kajala et al., 2011, Hibberd et al., 2008), (Carvalho et al., 2011, Parry et al., 2008) (Taniguchi et al., 2008, Leegood, 2002). In addition to increasing the maximum yield, overall yield can be increased by reducing sources of yield loss. This can be done by reducing loss from herbivory, abiotic stresses, lodging and pathogens.

One of the most important developments in wheat breeding was the adoption of semi-dwarfing Rht genes in the green revolution. This decreased yield loss by increasing biomass partitioning to the ear and significantly reducing the amount of lodging (Rebetzke et al., 2012). The shorter plant stature led to the stems being less likely to break in windy conditions. Dwarf lines produce a lower amount of yield than semi-dwarf lines (Flintham et al., 1997). This is because of trade-off between the different traits affected by the change in GA regulation. Whilst dwarf plants will have a reduced

frequency of lodging compared to semi dwarf lines. This is counteracted by the far greater reduction in grain development. When breeding plants to improve one trait, potential trade-offs with others need to be considered carefully (Brown and Rant, 2013). This does not mean that the trait cannot be improved though; ways of reducing lodging, without further altering height, are still being worked on. This can be done via changes in stem thickness and root structure (Reynolds et al., 2009, Berry et al., 2007).

Improving yield by reducing yield loss from disease is another key area of study. Pathogens destroy approximately 10% of all plant products worldwide (Strange and Scott, 2005). Breeding for disease resistance has shown great success in improving crops historically, however pathogens have adapted to previous control strategies (Bayles et al., 2000) . Identifying durable sources of resistance to pathogens and improving management and control of wheat diseases is an important goal for increasing yield in the future.

1.2 The 6A QTL for thousand grain weight and plot yield

To identify potential new targets for yield improvement and to characterise gene x environment interactions that contribute to yield, Snape et al (2007) studied yield traits with QTL analysis in several double haploid populations of wheat. They identified a previously unknown QTL for yield traits on chromosome 6A. This was identified in crosses of Spark x Rialto, Savannah x Rialto and Badger x Charger with multiple alleles having differential effects on plot yield (Snape et al., 2007). The QTL showed stronger association with increased grain weight than any other trait, indicating that this is the cause of the increased yield. This discovery of a new QTL for grain size is a useful finding for the improvement of yield, as grain size is a component of yield that had not shown any recent improvements (Brancourt-Hulmel et al., 2003, Shearman et al., 2005).

1.3 Introduction to septoria tritici blotch

Septoria tritici blotch (STB) of wheat is one of the most important wheat diseases in the UK. It is an caused by *Zymoseptoria tritici,* an ascomycete fungus that was previously known as *Mycosphaerella graminicola (*anamorph*, Septoria tritici)* (Quaedvlieg et al., 2011) . STB causes the formation of pale spots on the leaf that grow

into irregular brown lesions. Within these lesions pycnidia form that are visible as small round black dots. If STB infects the upper leaves of the crop it can cause severe damage and yield loss (King et al., 1983, Thomas et al., 1989, Shaw and Royle, 1989a). The yield loss is caused by septoria lesions reducing the amount of green leaf area on the flag leaf and second leaf and consequently reducing grain filling (Shaw and Royle, 1989a, Parker et al., 2004) If the STB causes damage early in the season, the yield loss may be caused by a reduction in number of grains as opposed to grain weight (Adolf et al., 1993).

Septoria tritici blotch is of global economic importance, with a large impact in temperate climates with high rainfall such as the UK (Fones and Gurr, 2015, O'Driscoll et al., 2014) Despite nearly 100% of UK crops being treated with fungicide to reduce the effect of STB, it still caused greater yield loss than any other disease of winter wheat between 1985 and 1989 (Cook et al., 1991).This is because even with recommended fungicide application, varieties with high levels of STB resistance typically lose 5-10% of wheat yield (HGCA, 2014). Susceptible varieties can have up to 50% of their yield lost during severe epidemics (Eyal et al., 1973).

1.4 Control of STB with fungicides

The cost of fungicide control for *Z. tritici* is estimated at $1.2bn (Torriani et al., 2015). However this cost is recouped in the increased wheat yield relative to not treating the crop. Four major groups of fungicides have been used to control STB: benzimidazoles, demethylation inhibiting fungicides (DMIs), strobilurin fungicides (QoIs) and carboximides (SDHIs). Resistance to benzimidazoles developed in 1984 and by 2002 populations of *Z.tritici* had developed the G143A mutation that made them resistant to strobilurins (Fraaije et al., 2003). This mutation has been shown to have subsequently occurred multiple times (Torriani et al., 2009). Isolates with strobilurin resistance have since become increasingly prevalent (McCartney et al., 2007) . Resistance has not yet developed for the DMI azoles. However, whilst not becoming fully resistant, fungal populations are becoming increasingly insensitive requiring higher doses of the fungicide to be effective (Cools and Fraaije, 2008). There is an overall trend to increased prevalence of less sensitive haplotypes and these are spreading

geographically (Brunner et al., 2008, Fraaije et al., 2007). This results in SDHI's and chlorthalonil being the only fully effective chemical treatments against STB. SDHIs are being increasingly used as the primary method of control against Septoria. Whilst no SDHI resistant isolates have been found in the field, mutagenesis work in labs has resulted in the development of resistance. As the selection pressure is increased by the greater use of the chemical, these mutations that lead to resistance will become more likely to occur in field conditions (Fraaije et al., 2012). The development of fungicide resistance is likely to be one of the reasons for the increase in importance of Septoria as a disease in the UK. Without the development of new ways to manage the disease this is likely to lead to large increases in yield loss to STB in the future.

1.5 Infection process of septoria tritici blotch

Septoria tritici blotch is caused by ascospores and pycnidiospores growing on a host leaf and then using hyphae to penetrate the leaf through the stomata. This initial growth requires high humidity. It produces no haustoria and remains intercellular; however large amounts of hyphae grow in the intercellular space if the host plant is susceptible. Despite the lack of distinct appressorium some hyphal tip swelling does occur at the point of stomata entry.(Shetty et al., 2003), (Siah et al., 2010))

Fungal growth occurs in the host tissues for approximately 4 weeks but this growth is symptomless for the first two (Keon et al., 2007).The length of the sympomless phase is variable, with temperature being an important contributing factor (Hess & Shayner 1987, Shaw, 1990). *Z. tritici* is frequently described as a hemibiotroph with the pre-symptomatic growth being considered biotrophic before a switch to a necrotrophic lifestyle at 10-14 days. There is no evidence of feeding from the host during this period. It operates more like an endophyte than a biotroph in this stage and it is proposed that it should be referred to as a "latent necrotroph" rather than a hemibiotroph (Sánchez-Vallet et al., 2015). The is based on the lack of specialised biotroph structures such as haustoria and arbuscules (Keon et al., 2007). It has been predicted that the nutrients in the apoplast should be sufficient to support growth of the fungus without additional feeding structures, (Spencer-Phillips, 1997) allowing an increase in size whilst not becoming a true biotroph.

The necrotrophic phase of the septoria infection appears to cause lesions by inducing programmed cell death of the host tissue. It has been speculated that septoria induces this response with some form of toxin, like similar wheat pathogens, *Stagonospora nodorum* and *Pyrenophora tritici-repentis* (Kema et al., 2008). Homologues of other pathogenic effectors have also been found within *Z.tritici* (Stergiopoulos et al., 2010). The interaction between the pathogen and the death of the host cells is unclear but recent work suggests that the septoria interacts with the host's chromatin via TaR1, delaying host cell death until it is ready to switch into necrotrophy (Lee et al., 2015).

1.6 Resistance to septoria tritici blotch

Disease control for STB is centred around keeping the flag leaf and 2nd leaf free from disease as they are responsible for the majority of the photosynthesis used for grain filling (Thomas et al., 1989, Shaw and Royle, 1989a). As previously discussed, fungicide application has been a key methodology for preventing major yield loss to STB. Weather predictions also play a key role in trying to direct this process, with fungicide spraying ideally occurring before predicted rain-fall when splash based spore transfer would be high. Given the increasing levels of fungicide resistance, the importance of alternative methods for controlling the disease is likely to increase in the future. There are no known examples of complete resistance to STB, instead varieties are considered resistance from the level of delay and restriction of disease development (Nelson and Marshall, 1990). Resistance to STB, like resistance to many diseases, can occur as major gene resistance and partial resistance. Major gene resistance refers to resistance that specifically acts against a particular isolate of STB and is controlled by one gene (Brading et al., 2002), whereas partial resistance is polygenic and causes a reduction in STB symptoms from multiple isolates (Zhang et al., 2001, Simon and Cordo, 1998, Chartrain et al., 2004a).

The characterisation and identification of genes for STB resistance has only occurred relatively recently. However by selecting for resistant phenotypes, they have been part of the breeding population for a long time. Out of the 16 major genes currently identified, (Stb1-12, 15-18) (Adhikari et al., 2004, Chartrain et al., 2009, Tabib Ghaffary

et al., 2012) Stb6 has been the easiest to study, and it has a classical gene for gene relationship (Brading et al., 2002).

The importance of Stb6 to control of STB in wheat has been studied in Chartrain et al (2005) and Arraiano et al (2009). These studies showed that presence or absence of Stb6 within existing varieties of wheat explained a significant amount of the cultivars' response to Septoria. It appears that various breeding programs have used lines with Stb6 as their source of resistance to STB, leading to it being present in many lines worldwide. Whilst identified as a major resistance gene, its important role in resistant lines indicates that Stb6 may also have a partial resistance effect, or be linked to partial resistance genes that are selected alongside it in most breeding programs.

Stb17 was identified in 2012 and only confers resistance on adult plants. The resistance in the adult lines was found with the isolate IPO88018 (Tabib Ghaffary et al., 2012). The degree of isolate specificity is not the same for all of the named Stb genes, with Stb16 providing good resistance to several isolates. Lines resistant to IPO88004 and/or IPO323 were found frequently, due to the prevalence of Stb15 & Stb6 in common breeding lines (Arraiano and Brown, 2006, Chartrain et al., 2004b, Arraiano et al., 2007). When considering wheat varieties, their resistance levels are unlikely to be due to one resistance gene, instead any given variety is likely to have a few partial resistance genes that set the basal level of resistance, then maybe a few Stb genes that lead to isolate specific resistance. For example Kavkaz-K4500 L6.A4 (KK) is a wheat variety with high levels of resistance, and is likely to have at least 4 major resistance genes, due to high levels of resistance to certain isolates. It is important to remember however that this does not make it highly resistant to Septoria in general, thus whilst a field of KK would suffer little damage if infected with IPO 323 or ISR 8036, it is quite susceptible to IPO90012 (Chartrain et al., 2004b). Major gene resistance is still useful because Septoria isolates are relatively confined geographically. Thus it could be possible to breed a variety with resistance for the most common isolates in a given country, as susceptibility to other isolates is unlikely to be a problem as the crop is unlikely to have to deal with them. However, this indicates that selection for major gene resistance could only be a short term solution as isolate movement does occur, and upon entering the region, a new isolate could cause a large amount of crop losses.

When considering breeding wheat that will be resistant to a pathogen at a long time scale, partial resistance is better target than major gene resistance. This is because there is a strong selection pressure for pathogens to evolve responses to major genes, and due to small sequence changes often having a large effect; it is relatively easy to do so. This can lead to arms races between the host and pathogen (Brown and Tellier, 2011). Experiments have shown Septoria isolates overcoming major gene resistance in the time frame of 3 years (Cowger et al., 2000). The development of new wheat cultivars can take a considerable period of time emphasising the need to develop lines that are durable. For partial resistance, the selection pressure is lower as pathogens without novel adaptations can still survive to reproduce. It is important to realise that the durability of resistance is dependent on how easily the pathogen can adapt to it and not due to loss of function in the host plant. It will be harder for the pathogen to adapt to changes in the defence signalling pathway for instance, than to changes in pathogen recognition. This is because in recognition events the pathogen has a direct influence on the interaction, making adaption easier. When breeding for resistance long term, the goal is to identify many sources of partial resistance and incorporate them all into the same variety, leading to high levels of non-specific resistance.

Identification of genes that affect partial resistance is more complex due to the differences in disease levels being continuous rather than the discrete “Resistant” vs “Susceptible” comparison used with major genes. Field trials have been used to assess the levels of resistance to Septoria tritici blotch, and in many instances, high levels of resistance occurs that is not caused by the presence of major genes (Arraiano et al., 2009, Kosellek et al., 2013). This allows the identification of new QTLs for studying partial resistance to STB. QTL analysis is an important technique in identifying sources of partial resistance. Crosses between resistant and susceptible varieties are made to try and identify any regions of the genome that are associated with resistance to multiple isolates. For example, QTLs for resistance were identified in the population Senat x Savannah (Eriksen et al., 2003). This association was only found in the resistance of adult plants to the disease, and was absent in seedling disease tests, suggesting that either this source of disease resistance changes with the age of the plant or is dependent on environmental factors not present in the seedling tests.

Ideally the aim would be to breed for varieties that had resistance throughout the life cycle of the plant, as reducing inoculum build-up in seedlings has an effect on the final levels of disease (Parker et al., 1999). However the economic impact of the disease is greatest during the development of the upper leaves so any reduction in STB in the adult leaves will also be important (Parker et al., 2004, Shaw and Royle, 1989a). This type of analysis has been used to identify many potential sources of Septoria resistance (Kelm et al., 2012, Risser et al., 2011, Kosellek et al., 2013, Simón et al., 2004). Some QTLs identified were isolate or environment specific; however some QTLs showed low levels of variation. Examples include the 5A, 6D and 7D QTLs found by Kosellek et al, (2013) and QTLs on chromosomes 3A and 6D from Risser et al, (2011). QTLs for partial resistance with low genotype x environment interactions are useful targets for breeding durable resistance.

1.7 Septoria tritici blotch and the 6A QTL

The focus of the project is on the relationship between levels of STB and yield at a QTL identified on chromosome 6A. The QTL was discovered as part of an association genetics study of 225 wheat cultivars (Arraiano and Brown, 2016). Association mapping works by including the population structure of the varieties in the analysis along with the genetic markers and STB data. The STB data used in the analysis comes from naturally infected field trials, and thus may identify both resistance and disease escape effects in the analysis. To control for this, data analysis incorporated plant height as a factor into the model with subsequent conclusions drawn from the adjusted STB scores accounting for height (AdjSTB). Of the markers identified in the analysis, Xpsp3071 on chromosome 6A explained most of the genetic variation (26.7%), leading to it to be selected for further study in this project.

The 6A marker identified as being associated with lower STB in the association mapping by Arraiano and Brown (2016) was associated with lower yield in the QTL study of Snape et al (2007). If there is a partial resistance gene at the 6A locus it appears to be linked closely to a gene that affects grain size. Identifying the two genes and would allow them to be decoupled from each other allowing the creation of lines with both the high yielding allele and the new source of resistance.

1.8 Disease escape

There are three main factors that determine the impact of disease on crop yield. These are disease escape, disease resistance and disease tolerance. Disease escape traits are those that reduce the movement and spread of the disease. Resistance refers to the ability of the plant to prevent the pathogen from causing disease. Finally the disease tolerance of a plant is its ability to succeed despite being infected with a disease. For example, if a plant is very tall and consequently infected lower leaves cannot spread spores to higher areas; the height of the plant is affecting disease escape. If a plant recognises a toxin produced by the pathogen and produces enzymes to break it down, it would be resisting the pathogen. A tolerance effect could be caused by an increased ability to partition starch into grain when stressed. This would lead to grain content being less adversely affected by the pathogen.

1.9 Primary infection of STB

Z.tritici is heterothallic and can only produce ascospores when two different mating strains interact. These airborne acospores are the main source of primary infection of seedlings with STB (Shaw and Royle, 1989b, Eriksen and Munk, 2003). Other factors may also have a minor impact, such as infected seed and transfer from alternative hosts (Brokenshire 1975).

The pseudothecia develop on dead leaf tissue a long time after the formation of the pycnidium, in the UK the time was shown as varying from 62-95 days, though work in the Netherlands indicates that it may be as low as 35 days (Kema et al., 1996b, Hunter et al., 1999). Pycnidiospores that have remained viable in the crop stubble can contribute to the primary infection of the next crop [Abrinbana et al., 2010, Djerbi, 1977).

It was proposed that the amount of surviving ascospores may affect the extent of STB epidemics in the following year (Daamen and Stol, 1992). Subsequent work showed that the amount of airborne ascospores was not a limiting factor, with sowing date being the main determinant of STB levels in the early growth stages of the plants (Morais et al., 2015).

1.10 Spore movement of STB

After the initial infection, the majority of subsequent transfer of pycnidiospores occurs via rainsplash (Shaw and Royle, 1986b, Hardwick et al., 2001). However when leaves are highly infected and spacing between infected and non infected leaves is low, spore movement can occur without the presence of splashy rainfall (Lovell, 1997). Even when rainfall is causing the transfer of the spores, it occurs at a much higher rate for short distances with a 5 fold reduction in spore transfer for 10cm of height (Shaw, 1987). Rain-splash can lead to both horizontal transmission of the pathogen (transferring it within a leaf layer) and vertical transmission (transferring the pathogen from lower leaves to upper leaves). Vertical transmission occurs less often but is necessary for epidemics to affect the upper leaves of the plant. However once some spores have reached the flag leaf, the extent of secondary multiplication and horizontal transmission seems to be the main determinant of final disease levels (Shaw and Royle, 1993).

Due to the importance of heavy rainfall and short distances for spore transfer, there is a strong relationship between the degree of STB and the amount of rainfall during stem extension (Polley and Thomas, 1991). Due to the importance of weather on STB development it is predicted that climate change may reduce STB severity (Gouache et al., 2013).

1.11 Disease escape traits affecting STB

Studies into how different traits affect disease escape have been performed on many different crop-pathogen systems (Madden and Ellis, 1990, Ntahimpera et al., 1998, Soleimani et al., 1996). Regardless of the species, the principles are the same. If a trait reduces the likelihood of successful spread between infected and uninfected material, it is a disease escape trait. For example, a more open canopy in bean cultivars was found to reduce levels of white mould due to the drier microclimate (Blad et al., 1978). A similar effect of openness of the canopy has also been found to reduce disease levels of apple scab in apple trees (Simon et al., 2006).

Height is a very important characteristic when considering disease escape and is especially important in splash-borne diseases such as Septoria. Increased plant height and increased height of individual leaves are associated with reduction of STB in the upper canopy, given the same initial level of infection by ascospores (Danon et al., 1982). Damage from STB occurs to a greater extent in short varieties due to more efficient transfer of spores up the plant. The widespread adoption of semi-dwarfing and dwarfing lines after the green revolution are suspected to have caused the increase in the importance of STB as a disease in the same time period (Baltazar et al., 1990). This relationship works on the basis that shorter plants will have greater amounts of inoculum reach them due to the distances between splash-event being reduced (Bahat et al., 1980). Thus the distance between the leaves is an important factor as it allows a ladder effect, wherein the disease moves up the plant one leaf at a time(Eyal, 1981).

Another factor that is might be predicted to effect transmission of the disease is crop spacing. Greater crop densities have been shown to increase the spread of another splash-borne disease *Pyrenopeziza brassicae* in oil seed rape(Pielaat et al., 2002). In work by Tompkins et al (1993) higher plant density affected the microclimate around the leaves, increasing leaf wetness, creating more favourable conditions for infection and increasing levels of STB. However work by Baccar et al (2011) showed no strong differences between density treatments for Septoria.

Lovell et al (1997) presents the argument that erect leaves will increase spread of STB by lowering the distance between established infected material and newly emerging leaves. However Arraiano found that more varieties with more erect leaves had less Septoria tritici blotch (Arraiano et al., 2009). This could operate via more prostrate leaves being hit by rain easier, or by the prostrateness being associated with increased length of the leaves, or susceptibility itself.

Heading date is also known to show significant association with levels of STB. This is due to earlier emerging leaves having longer to develop STB symptoms before they are scored (Van Beuningen and Kohli, 1990). However this association between heading

date and levels of STB is not seen in every experiment that includes heading date in the analysis (Arraiano et al., 2009, Arraiano and Brown, 2015, Simón et al., 2005).

1.12 Disease escape vs resistance at the 6A QTL

Disease escape or resistance

The analysis performed in Arraiano and Brown (2016), accounts for disease escape by including the effect of height in the model. This leaves two possibilities for the reductions in AdjSTB levels; they are caused by a resistance gene in the region or by an uncharacterised escape trait.

Linkage or Pleiotropy

The reduction in Septoria tritici blotch associated with the 6A QTL may be caused by the same genes as the 6A QTL yield effect or they may just be closely genetically linked. From a plant breeding perspective, genetic linkage would be preferable as it would allow the breeding of varieties with both the higher grain weight and lower STB. However, the metabolic changes involved in a novel resistance gene may cause trade-offs between other pathways and thus directly influence the yield. Alternatively if changes in leaf development and morphology decrease STB by altering disease escape, this may also be intrinsically linked to the yield.

Escape traits and Yield

Reducing disease severity by encouraging disease escape has the advantage of not enabling an arms race with the pathogen, leading to more stable benefits over time. However escape traits can be undesirable, because they can be maladaptive in terms of agronomic properties and yield. For example, tall crops would have greater disease escape than semi-dwarf varieties, but the other advantages of growing semi-dwarf lines means that varieties with a tall stature are rarely grown in modern farming. This may not just apply to this specific yield trade off; other unexamined escape effects may cause a trade off between yield and escape from STB.

2 General Materials and Methods

2.1 Line selection

The varieties of wheat selected for use in these experiments were based on the results from the STB association genetics study in Arraiano and Brown (2016). The microsatellite marker Psp3071, which had four major alleles, mapped very close to the locus on chromosome 6A which affected STB. All other alleles discovered in this region were present in fewer than five lines. To study the effect that this region has on the levels of STB, varieties were selected to create near isogenic lines (NILs) with contrasting alleles. The greatest difference in STB levels was between simple-sequence repeat (SSR or microsatellite) alleles with fragment sizes 161 bp and 167 bp, associated with high and low levels of STB respectively. An existing cross of Flame (allele 167) and Longbow (allele 161) was used to study the effect of the Psp3071 region of the genome on STB (Brading et al., 2002).

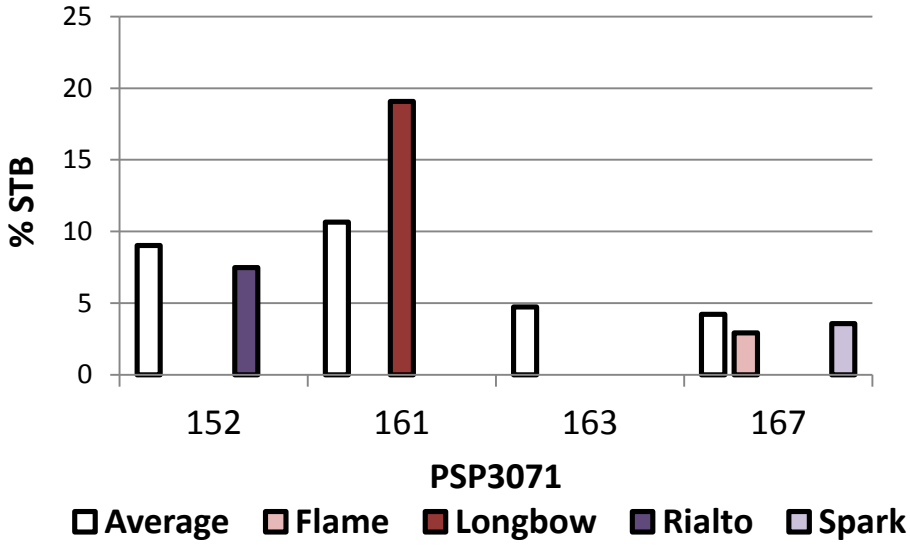


Figure 2.1.1: STB data from association genetics study. Adapted from the Supplementary figures of (Arraiano and Brown, 2016). Values shown are the % STB scores from the four selected varieties compared to the average STB scores across other varieties with the same psp3071 allele.

Crosses between Spark and Rialto were also selected for study due to Spark x Rialto crosses already being used at the John Innes Centre to study the effect Psp3071 region

on Chromosome 6A has on yield (Snape et al., 2007); work on this locus is continuing in the lab of Dr C. Uauy. Spark shares the low disease allele Psp3071-167 and Rialto has an allele with fragment size 152 bp at the locus (Figure 2.1.1). The data from Arraiano and Brown (2016) shows that these two alleles should have significantly different levels of STB, making the material also suitable for studying the locus.

2.2 Flame x Longbow material (FLLO).

The Flame x Longbow lines were generated from the population made by Brading et al (2002). Two different families bred from independent F2 plants were studied as part of this population, family 16 and family 24. Existing F3 lines were selfed by a single-seed descent process. At the F6 generation, lines from both families were genotyped for the SSR/microsatalite maker Psp3071. Heterozygotes for this marker were selected, selfed to F7 and taken forward by single seed descent to the F8 generation. This work was performed prior to my arrival on the project by Lorelai Billham.

The use of the two distinct families allows effects to be tested in multiple backgrounds. The lines have different combinations of Flame and Longbow material across their chromosomes, but between the NILs within the individual families, variation should be low in the majority of the genome, with the greatest difference being in the region around the marker.

Initial selection

The F8 Flame x Longbow plants were selfed to produce a population with homozygotes of both alleles and heterozygotes (Hets) at Psp3071 (Figure 2.2.1). These lines were initially characterised using the psp3071 SSR marker. Genotyping using the SSR marker proved to be time consuming and inefficient. Therefore to genotype the lines in more detail, work was switched to using KASPAR markers (Chapter 2.7). Identifying KASPAR markers that could be used to genotype the lines was complicated by the fact that whilst SNPs for 6A in Longbow were known, Flame had not had SNPs identified in the same process. So when trying a marker in the region of interest, there was a high chance that the marker would not be different for the parental lines, and would thus be unsuitable for use in genotyping the NILs. Suitable markers were found for the region (Table 2.2.1) which aligned with the psp3071 data in identifying which of the

plants being grown were Hets, Flame or Longbow, at the 6A locus. Full data on each line tested with these markers is given in the appendix (Table 8.1).

Marker	Marker ID	Position CM (AxC)	Polymorphic	FLLO NILs
BS00003881	13	95.88	Y	N
BS00004377	12	95.88	Y	N
BS00022947	M5	97.00	Y	N
BS00022992	M2	98.13	Y	N
BS00001132	14	99.82	Y	Y
BS00003581	18	99.82	Y	Y
BS00009783	17	99.82	Y	Y
BS00009871	15	99.82	Y	Y
Psp3071			Y	Y
BS00009988	16	99.82	Y	Y
BS00023089	M3	99.29	Y	Y

Table 2.2.1: Markers used in the initial genotyping. A Y in the polymorphic column means that the marker being used showed differences between the Flame parent and Longbow parent. A Y in the FLLO NILs column means that the Flame x Longbow NILs have differences at this marker.

This first set of genotyping of the lines was performed on all plants grown in 2012. Further material for study was generated by bagging the plants grown as part of the 2012 environment 54 experiment. This led to selfing of the homozygous lines creating F9 seed suitable for use in the 2013 experiments. The system used to refer to lines of the plant was based on the plant chosen in the preceding generation (subfamily) and the number assigned to the plant in this experiment. For example, line 16A3, is from family 16, subfamily A, and was in pot 3 in the 2012 G54 experiment. The homozygotic seed was then bulked in a small field trial to generate enough seed for the 2014 & 2015 experiments. Seed multiplication was performed in conjunction with the John Innes Centre field trials team at Church Farm, Bawburgh. The lines used are marked in Table 8.1. At the end of the season, the plots were harvested and produced about 4kg of seed.

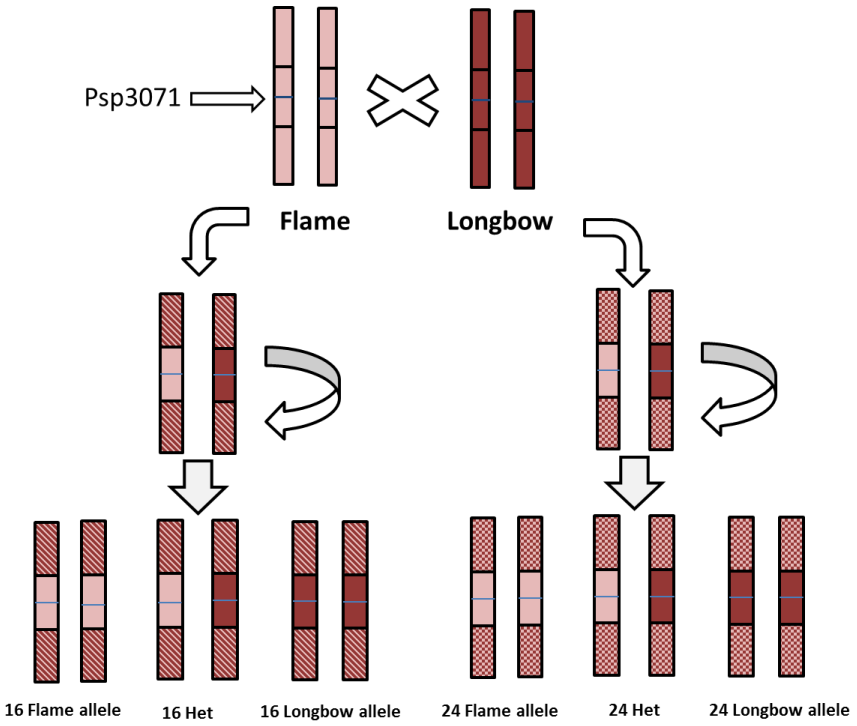


Figure 2.2.1: Simplified Flame x Longbow population structure. The FLLO NILs were generated from crossing the two varieties, and then selected for variant alleles at Psp3071. Two families were taken forward to the F8 generation. These were genotyped to identify homozygote plants for the Flame and Longbow alleles at the 6A locus in both families. These are referred to as the FLLO NILs.

Main Lines

Having established a set of Flame x Longbow NILs that are homozygotic for the relevant markers at the 6A locus, only a subset of daughter lines of the F8 lines was selected for further study. It was decided that it would be preferable to have replicates of the same lines in different blocks and environments rather than measuring lots of lines only a few times. Eight FLLO lines were chosen so that each family and allele combination had two different lines (Table 2.2.2). These were chosen from the early genotyping data as lines that had always been clear in their marker results and repeats so that the likelihood of them being misidentified was as low as possible. These eight lines were not only used in all of the subsequent field trials but also the 2014 and 2015 plastic glasshouse work and related experiments.

Cross	Family	Subfamily	6A Allele	Line name	Line Code
Flame x Longbow	16	16A	Flame	16A3	16 F A
Flame x Longbow	16	16A	Longbow	16A4	16 L A
Flame x Longbow	16	16B	Flame	16B5	16 F B
Flame x Longbow	16	16B	Longbow	16B13	16 F L
Flame x Longbow	24	24C	Longbow	24C15	24 L C
Flame x Longbow	24	24C	Flame	24C16	24 F C
Flame x Longbow	24	24D	Longbow	24D1	24 L D
Flame x Longbow	24	24D	Flame	24D16	24 F D
Spark x Rialto	1		Rialto	Bc4-4	4
Spark x Rialto	1		Spark	Bc4-6	6
Spark x Rialto	1		Rialto	Bc4-7	7
Spark x Rialto	1		Spark	Bc4-9	9
Spark x Rialto	1		Rialto	Bc4-11	11
Spark x Rialto	20		Spark	Bc4-22	22
Spark x Rialto	20		Rialto	Bc4-26	26

Table 2.2.2: The 15 main near isogenic lines selected for detailed study

For these eight lines chosen for further study, additional Kaspar markers were identified to further characterise the region. The results of this are shown in Table 2.2.3 with each data point having been replicated on multiple plates and from DNA extracted from different plant material.

Marker	138 383	140 616	160 910	022 992	183 332	117 196	125 902	139 645	144 445	149 380	154 514	170 101	023 089	180 917	184 052	184 342	184 784	140 050	181 736	139 857	138 275	179 073	182 784	138 349	155 487	175 896	175 897
Order	535	537	541	546	566	679	685	701	710	717	718	732	740	746	761	762	765	883	888	930	963	1,039	1,040	1,074	1,121	1,125	1,126
CM order	74	74	74	74	74	79	79	79	79	79	79	79	79	79	79	79	79	79	79	80	82	84	84	85	85	85	85
Flame	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
Longbow	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
16A3	L	L	L	L	L	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	L	L	L	L	L	L	L
16A4	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
16B5	L	L	L	L	L	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	L	L	L	L	L	L	L
16B13	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
24C15	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
24C16	L	L	L	L	L	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	L	L	L	L	L	L
24D1	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
24D16	L	L	L	L	L	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	L	L	L	L	L	L

Table 2.2.3: Additional marker work on main FLLO lines. The SNP markers used are listed without the prefix BS00. The CM order shows the known information on the position of the markers in centimorgans. The Order factor was from the marker database, and shows the relative position of markers within the range. Order is shown reduced by 29000 to ensure they fit in the table. The markers were selected from an iselect database generated for the use of the John Innes centre crop genetics department. From the list of available markers on chromosome 6A, 172 were sent for analysis by Ricardo Ramirez Gonzales. This process allowed identification of 62 markers that were chromosome specific and non homeologous. These markers were run on the Flame and Longbow parental lines and the eight main FLLO NILs, and the results from 27 of these markers are shown. The data from the other markers tested are not shown due to them either failing to work, or due to them being much further away from the NIL.

2.3 Spark x Rialto Material

The Spark x Rialto material was developed by James Simmonds and Nick Bird from the Uauy lab at the John Innes Centre. Their material was developed by selecting the 6A region with Psp3071 and Kaspar markers located close to it such as BS00009871. The Spark x Rialto NILs were generated by backcrossing double haploid lines that were homozygous for the Rialto allele across the region of interest with the Spark parent. These lines were then advanced to BC2 & BC4. They were then selfed to identify NILs homozygous for each allele on chromosome 6A from the resultant $BC2F_2$ and $BC4F_2$ plants. Additional detail on the generation of this population and its mapping can be found in Simmonds et al (2014).

Three different sets of Spark x Rialto material were used in the experiments reported here. BC2 NILs were used when SPRI lines were included in the 2012 and 2013 experiments, but the BC4 lines were available in late 2013 and were subsequently used for the majority of the experiments. As with the Flame x Longbow material, eight Spark x Rialto lines were selected as the main experimental lines. However one of the Spark lines used became heavily contaminated with bunt (*Tilletia tritici*) and had to be discarded. Combined with the FLLO material, this leads to there being 15 main lines used in most experiments (Table 2.2.2). Marker data for the 6A region in the 7 main lines used is shown in Table 2.3.1.

A population of SPRI recombinant lines were developed by the lab of Dr. C. Uauy for narrowing down the interval being studied. In this population, SPRI 10C and 9C have no change in their recombination compared to the Spark and Rialto alleles in the BC4 NILs respectively**.** Another nine lines were studied that break up the interval into regions with different alleles (Table 2.3.2). These lines were used in the 2014 and 2015 plastic glasshouse experiments and the 2015 field trials.

Marker	BS000 023192	BS000 003635	BS000 09584	BS000 10933	BS000 66522	BS000 22057	BS000 22992	BS000 03881	wmc32	psp3071	BS000 03581	Ta GW2	BS001 05973	BS000 72146	BS000 09871	BS000 09988	BS000 10730	BS000 22836	BS000 22613	BS000 23020	barc113	BS000 03835	gwm570
Hv CM			23.37			49.07	49.22	49.78					54.89	55.84	53.60		64.44	70.50	72.16	88.59		94.90	
Spark	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Rialto	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
BC4-6	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
BC4-9	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
BC4-22	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
BC4-4	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
BC4-7	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
BC4-11	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
BC4-26	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

Table 2.3.1: Genotyping data for the 6A region for the selected SPRI NILs. Hv CM gives the relative position of the markers in centimorgans. The markers shown are a subset of those tested on the 6A NILs. These results were provided by James Simmonds who created this population. Further information on these NILs can be found in Simmonds et al (2014).

Marker	BS00 023192	BS00 003635	BS00 009584	BS00 010933	BE5178 58	BS00 066522	BS00 022057	BS00 022992	BS00 008881	wmc 32	psp30 71	BS00 008581	Ta GW2	BS00 105973	BS00 072146	BS00 009871	BS00 009988	BS00 010730	BS00 022836	BS00 022613	BS00 023020	barc1 13	BS00 003835	gwm5 70
Hv CM			23.37				49.07	49.22	49.78					54.9	55.84	53.60		64.44	70.50	72.16	88.59		94.90	
Spark	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Rialto	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
SR6	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
SR21	S	R	R	R	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
SR12	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	S	S
SR9	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S
SR9c	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
SR30	S	S		R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S
SR17	S	S	S	S	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
SR15	S	S	S	S	S	S	S	S	S	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R
SR14	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R	R	R	R	R
SR10	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R	R
SR10c	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Table 2.3.2: Genotyping data for the 6A region in SPRI Recombination lines. Hv CM gives the relative position of the markers in centimorgans. The markers shown are a subset of those tested on the 6A NILs, selected to show the differences between the recombinants. These results were provided by James Simmonds who created this population.

2.4 Environments

Reference	Environment	Year	Location	Type
S63 2012	S63	2012	JIC	Heated Glasshouse
S54 2012	S54	2012	JIC	Glasshouse
S54 2013	S54	2013	JIC	Glasshouse
S53 2013	S53	2013	JIC	Glasshouse
Plastic Glasshouse 2014	North 1 B	2014	JIC	Plastic glasshouse
Morley 2014	Morley	2014	Norfolk	Field trial
Hercules	Hercules	2014	Norfolk	Field trial
Track	Track	2014	Norfolk	Field trial
Ragt	Ragt	2014	Cambridgeshire	Field trial
Limagrain	Limagrain	2014	Cambridgeshire	Field trial
Syngenta	Syngenta	2014	Dorset	Field trial
Teagasc	Teagasc	2014	Carlow	Field trial
Plastic Glasshouse 2015	North 1 B	2015	JIC	Plastic glasshouse
Morley 2015	Morley	2015	Norfolk	Field trial

Table 2.4.1: List of environments used in the project

Various environments were used for experiments (Table 2.4.1).Key features of each experimental set up and plot layouts when relevant are listed below.

S63 2012

30 plants were grown for each available subfamily (16A, 16B, 24A, 24B, 24C, 24D). These plants were sown prior to genotyping of the lines, 94 of the 180 plants were subsequently identified as homozygotes for the 6A alleles. Plants were initially sown in trays of 60 plants within their subfamilies. Each plant was potted up into 1 L pots then randomised, but not into set blocks. Watering was provided by the Horticultural Services staff at JIC.

S54 2012

Six plants were grown of the 16A sub family and 18 plants sown of the other 5 subfamilies. The genotyping data for these plants is shown in the appendix (Table 8.1) Plants were grown in 1 L pots in no set blocks. Watering was provided by the Horticultural Services staff at JIC.

S54 2013

Each of the following 19 lines had 9 plants grown in this environment. 16A1,16A2,16A3,16A5,16B5,16B8,16B9,24B7,24B6,24B18,24C1,24C2,24C4,24C7,24C16,24D1,24D2,24D3 and 24D17. Plants were grown in 1ltr plots and were randomised without set blocks. Watering was provided by the Horticultural Services staff at JIC.

S53 2013

This environment was selected because it was a soil glasshouse, allowing the experiments to be performed at ground level as per in a polytunnel or field. This was necessary for the prototype escape experiment (Esc0) and made infecting and scoring the resistance tests more practical.

The glasshouse was unheated but temperature was controlled by performed by having vents on the side of the glasshouse open and shut to try and prevent extreme oscillations in temperature. Watering was provided via timed hydration of matting underneath the crops. As temperatures increased throughout the year the timings were altered accordingly. Additional manual watering was performed every 2-3 days. This was done differently per plot in the escape experiment.

This environment was split into three sections. The first was the preliminary escape test, the second was the first adult plant resistance test (AP1) and finally additional plants of all included lines were grown in smaller blocks for physiological tests. The layout of the environment is shown in Figure 2.4.1.

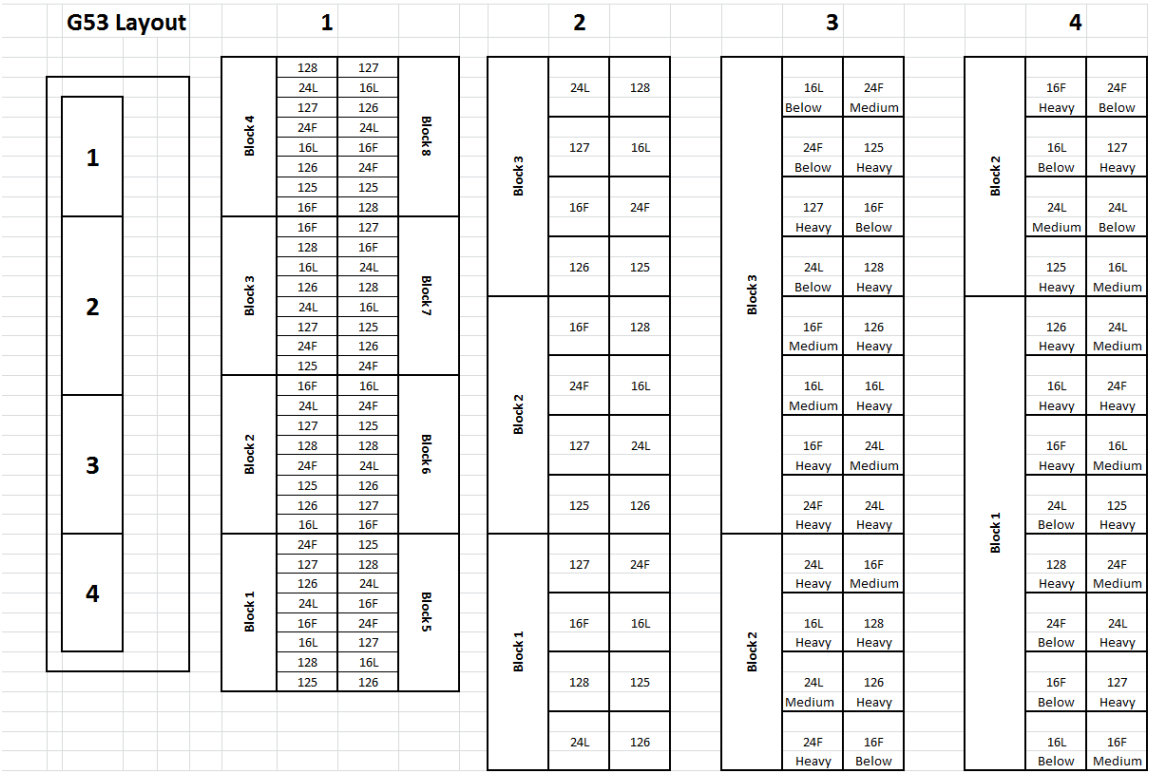


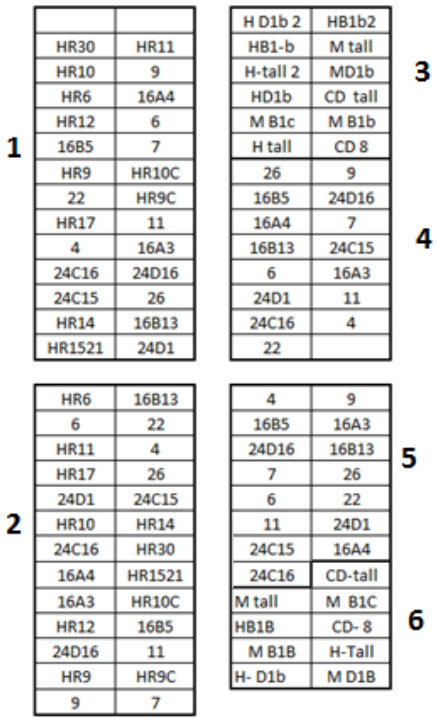
Figure 2.4.1: Plot layout of G53 2013. This trial was organised into three experiments. Section one consisted of eight blocks containing four plants of each of the eight lines, for measuring physiological and developmental traits. Section two had three blocks, each containing eight plots of sixteen plants. These plots were used for testing adult plant resistance. Section three is the plots used for Esc0, a preliminary study into the viability of testing disease escape experimentally. It is split into two figures (3&4) above for ease of viewing only. The work in G53 used eight different lines. The four SPRI lines used were BC2 NILs with the odd numbers being the lines with the Spark allele. The four FLLO lines used were 16A3 (16F), 16B8 (16L), 24C15 (24L) and 24D16 (24F). Heavy, Medium and Below refer to the type of watering used in the plot, corresponding to watering with a sprinkler, misting and additional watering at the base respectively.

Plastic Greenhouse 2014

The plot layout for the plastic greenhouse experiments in 2014 is shown in Figure 2.4.2. This environment is a great compromise between working in a glasshouse and a

traditional polytunnel; because, the large open space and higher humidity make it an ideal environment for pathology experiments. The environment has a built in ventilation systems for controlling temperature. As part of the design of the experiments, watering was performed via a roof mounted irrigation system as well as standard ground level matting (Figure 2.4.3).

The environment was split into six sections. The bottom half of the space was used for escape tests and the top half for studying flag leaf resistance and physiological and developmental traits. Each half was subsequently split into two sections with one including SPRI recombinant lines and the other being infected with a different isolate and split into a repeat of the selected 15 lines and the *Rht* NILs experiments. Within these sections there were two columns of plots referred to as the left and right side (Figure 2.4.4).



HR30	HR11
HR10	9
HR6	16A4
HR12	6
16B5	7
HR9	HR10C
22	HR9C
HR17	11
4	16A3
24C16	24D16
24C15	26
HR14	16B13
HR1521	24D1

1

H D1b 2	HB1b2
HB1-b	M tall
H-tall 2	MD1b
HD1b	CD tall
M B1c	M B1b
H tall	CD 8
26	9
16B5	24D16
16A4	7
16B13	24C15
6	16A3
24D1	11
24C16	4
22	

3

4

HR6	16B13
6	22
HR11	4
HR17	26
24D1	24C15
HR10	HR14
24C16	HR30
16A4	HR1521
16A3	HR10C
HR12	16B5
24D16	11
HR9	HR9C
9	7

2

4	9
16B5	16A3
24D16	16B13
7	26
6	22
11	24D1
24C15	16A4
24C16	CD-tall
M tall	M B1C
HB1B	CD- 8
M B1B	H-Tall
H- D1b	M D1B

5

6

Figure 2.4.2: Plastic glasshouse plot randomisation 2015. Six separate randomisations were performed for the different sections. 1) AP2 test, block 1, 2) Esc1 test with isolate CHC3, 3) Rht adult plant resistance test, 4) AP2 test block 2, 5) Esc1 test with isolate JIC040 & 6) Rht escape test. Sections one and two used the main 15 NILs and the SPRI recombinants. Sections four and five just used the main 15 FLLO and SPRI NILs.

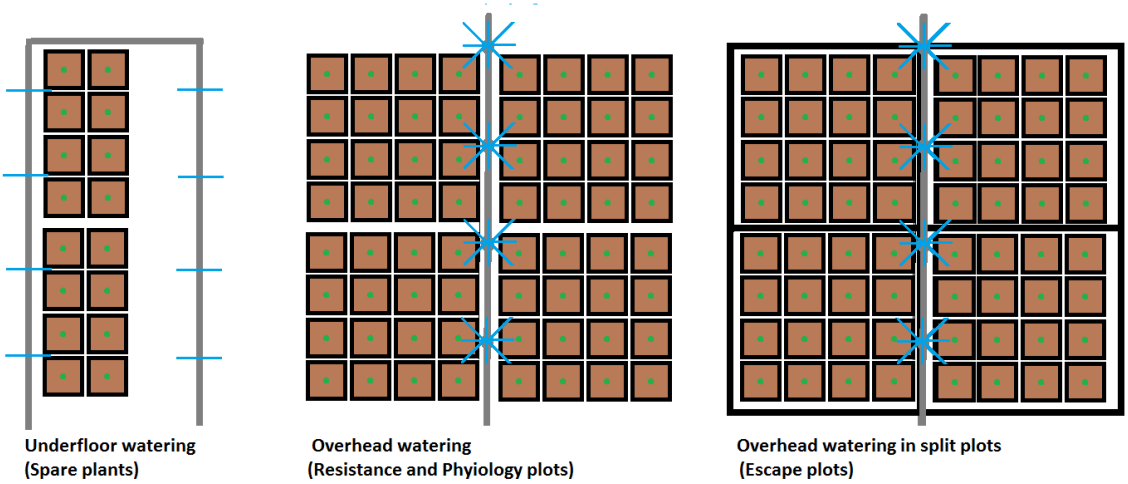


Figure 2.4.3: Types of watering in the plastic glasshouses. Three types of watering set up were used in the plastic glasshouse experiments. Ground level watering via matting was used along the edge of the tunnel for multiplying seed and growing spare plants. The majority of plots were watered via an overhead sprinkler system. Pipework in the roof of the tunnel had nozzles to spread water at regular intervals along the pipe. Testing confirmed that this gave an even spread of water on either side of the pipe. Plots were arranged so they were equidistant from this central pipe. For the escape plots, barriers were placed around the plots.

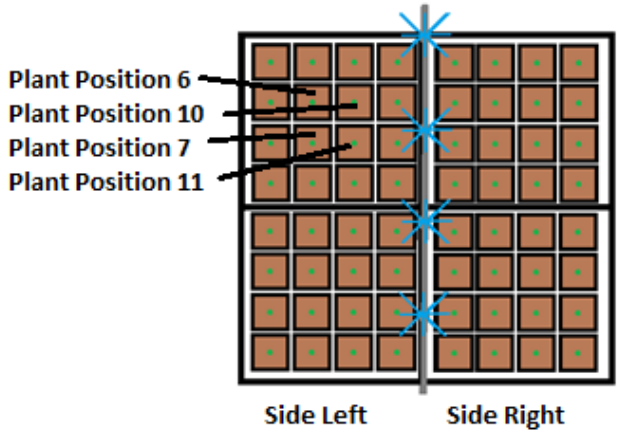


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Figure 2.4.4: Positional factors in plastic glasshouse plots. When analysing the data from plastic glasshouse experiments the positional factors of side and plant position can be included. The factor of “Side” refers to the plots position relative to the overhead watering

Plastic glasshouse 2015

The 2015 plastic glasshouse experiments were performed to repeat the 2014 experiments, thus the environment was arranged in a similar manner. There were two main differences in environmental set up. The first was that the pipes for ground level watering were laid underneath the main plots. These pipes were unconnected for the majority of the experiment, but allowed for more even watering when overhead watering had to be disconnected. This occurred when leaves were inoculated with the fungus or sprayed to keep other diseases away. Secondly the plot arrangement was also randomized for the new material (Figure 2.4.5).

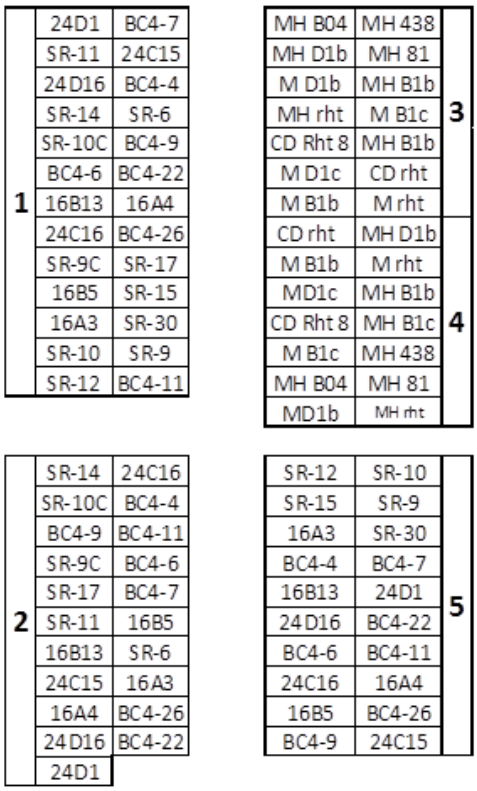

Figure 2.4.5: Plastic glasshouse plot randomisation 2015. Section 1 was the 2015 tests of physiology on the SPRI recombinants and main lines. The same lines were used in section 2 and 5 for the Esc2 escape test.

Field trial plot layouts

Field trials were run in 2014 and 2015 and were used to measure traits selected from the glasshouse work in field conditions. The trials were either treated with fungicides to measure yield traits (Y) or encouraged to develop STB (S). The trial plans for the field trials ran by the John Innes Centre are shown below (Figure 2.4.6 -2.4.12). Exact plot plans are not available for the trials run by collaborators as the lines were often included as part of larger trials they were running. A summary of the lines sent for testing by each collaborator is included (Table 2.4.2). Each block in the 2014 yield trials had one line repeated an additional time as having sixteen plots rather than fifteen aided trial design and improved overall replication.

Block 1			Block 2		
Row 1	**Row 2**	**Row 3**	**Row 4**	**Row 5**	**Row 6**
Claire Solstice	BC4-7	Sol-Hum	Claire-Diego	Claire Podium	JB-Diego Humber
BC4-26	KWS Podium	JB Diego	Sol-Pod	Sol-Diego	Claire Solstice
Sol-Diego	Claire-Diego	16 F A	BC4-4	16 F B	Bel-hum
JB-Diego Humber	Solstice	Beluga podium	Claire	BC4-7	JB-Diego Beluga
24 L D	Claire	16 L A	24 F D	24 L C	Claire-Bel
JB-Diego Beluga	Claire-Hum	Claire-Bel	24 F C	BC4-22	Claire-Hum
Humber	24 L C	BC4-4	Solstice	Humber	Sol-Bel
Claire Podium	Sol-Bel	24 F D	Hum-Pod	BC4-26	16 L B
BC4-11	BC4-9	Hum-Pod	16 F A	24 L D	Beluga
Diego-Pod	Sol-Pod	16 F B	BC4-11	BC4-6	Beluga podium
Beluga	BC4-6	24 F C	Diego-Pod	Sol-Hum	BC4-9
16 L B	BC4-22	Bel-hum	16 L A	JB Diego	KWS Podium

Figure 2.4.6: Hercules (S) plot randomisation 2014. Design consisted of two randomised blocks of the selected lines. The 6m^2 plots include each of the main NILs selected for further study (Table 2.2.2).

Row 3	Row 2	Row 1	
Sol-Diego	KWS Podium	Claire Solstice	Block 2
Sol-Pod	Claire-Diego	Humber	
Sol-Hum	Claire-Hum	BC4-26	
24 F D	24 L C	Claire Podium	
Claire	16 L B	Solstice	
Claire-Bel	24 F C	Sol-Bel	
JB-Diego Beluga	Diego-Pod	BC4-9	
Beluga	BC4-11	BC4-4	
BC4-6	JB Diego	BC4-7	
Hum-Pod	BC4-22	Beluga podium	
16 F A	JB-Diego Humber	16 F B	
24 L D	Bel-hum	16 L A	
JB-Diego Beluga	16 L A	Sol-Hum	Block 1
16 F B	Bel-hum	Claire-Hum	
BC4-22	JB Diego	Claire-Bel	
Sol-Bel	Humber	Diego-Pod	
BC4-6	24 L D	Beluga	
Sol-Diego	Solstice	BC4-7	
KWS Podium	24 L C	Claire-Diego	
Sol-Pod	24 F D	BC4-4	
Claire Solstice	BC4-9	Hum-Pod	
16 F A	24 F C	Claire	
BC4-11	16 L B	BC4-26	
Beluga podium	Claire Podium	JB-Diego Humber	

Figure 2.4.7: Track (S) plot randomisation 2014 Design consisted of two blocks of the selected lines. The 6m^{2}plots included each of the main NILs selected for further study (Table 2.2.2).

	Block 1	Block 2	Block 3
Row 16	BC4-4	BC4-26	BC4-11
Row 15	16 F A	24 L C	BC4-4
Row 14	24 L D	BC4-6	BC4-22
Row 13	BC4-7	16 F B	24 L C
Row 12	BC4-22	16 L A	16 L A
Row 11	24 F D	16 L B	BC4-6
Row 10	BC4-6	BC4-7	16 F A
Row 9	BC4-4	24 L D	BC4-9
Row 8	16 F B	BC4-11	24 F C
Row 7	24 F C	BC4-9	BC4-26
Row 6	BC4-11	24 F C	24 F D
Row 5	24 L C	24 F D	BC4-7
Row 4	16 L A	BC4-22	BC4-22
Row 3	BC4-26	BC4-4	24 L D
Row 2	16 L B	BC4-6	16 L B
Row 1	BC4-9	16 F A	16 F B

Figure 2.4.8: Track (Y) plot randomisation 2014 Design consisted of three blocks of the selected lines. The 6m^{2}plots included each of the main NILs selected for further study (Table 2.2.2). Due to the field layout, this trial was split, with another trial being placed between Row 8 and Row 9.

	Row 1	Row 2	Row 3
Block 1	BC4-6	KWS Podium	Sol-Bel
	24 F C	24 L C	Sol-Hum
	BC4-7	Claire-Bel	24 L D
	Humber	BC4-4	16 L A
	Claire Solstice	16 L B	Claire-Hum
	BC4-22	24 F D	Sol-Diego
	Diego-Pod	BC4-11	Sol-Pod
	JB Diego	Beluga podium	BC4-26
	16 F B	BC4-9	Hum-Pod
	JB-Diego Humber	Beluga	Claire-Diego
	16 F A	Claire Podium	Claire
	Bel-hum	JB-Diego Beluga	Solstice
Block 2	Sol-Bel	24 L D	Claire Podium
	Claire	BC4-6	16 F A
	Hum-Pod	Sol-Hum	24 F C
	Beluga	BC4-7	24 L C
	24 F D	Bel-hum	Solstice
	BC4-22	KWS Podium	Sol-Pod
	Claire-Diego	BC4-4	JB-Diego Humber
	Sol-Diego	BC4-26	Diego-Pod
	16 F B	Claire-Hum	JB-Diego Beluga
	Beluga podium	BC4-11	Claire Solstice
	Claire-Bel	16 L A	16 L B
	BC4-9	JB Diego	Humber

Figure 2.4.9 Morley (S) plot randomisation 2014 Design consisted of two blocks of the selected lines. The 6m^{2}plots included each of the main NILs selected for further study (Table 2.2.2).

Block 1	Block 2	Block 3
BC4-9	BC4-26	24 F C
BC4-7	24 L C	BC4-7
24 L C	BC4-6	24 F D
24 L D	BC4-22	BC4-6
16 F A	16 F B	24 L D
BC4-22	BC4-7	16 F A
16 F B	BC4-11	16 F B
16 L B	BC4-4	24 F D
24 F D	16 F A	24 L C
BC4-11	16 L A	16 L B
16 L B	24 L D	BC4-9
BC4-26	24 F C	BC4-11
BC4-4	BC4-9	BC4-22
BC4-6	24 L D	BC4-4
16 L A	16 L B	BC4-26
24 F C	24 F D	16 L A

Figure 2.4.10: Morley (Y) plot randomisation 2014. Design consists of three blocks of the selected lines. The 6m^{2}plots include each of the main NILs selected for further study (Table 2.2.2).

Row 1	Row 2	Row 3	Row 4	Row 5	Row 6	Row 7	Row 8	Row 9
24 L D	HR-SR10-C	16 L A	HR-SR30	BC4-22	Spark	16 F B	BC4-22	BC4-7
HR-SR17	Spark	HR-SR12	HR-SR10	HR-SR10	16 L A	24 L D	24 L C	16 F B
BC4-11	BC4-6	16 F A	BC4-7	24 L C	HR-SR15	Rialto	16 F A	BC4-6
24 F C	16 F B	HR-SR15	24 F D	HR-SR21	HR-SR6	HR-SR10-C	BC4-26	16 L A
Guard	Guard	Guard	Guard	Guard	Guard	Guard	Guard	Guard
BC4-26	HR-SR14	HR-SR9	BC4-4	16 F A	BC4-11	HR-SR9-C	BC4-4	BC4-11
BC4-9	BC4-4	16 L B	HR-SR9	HR-SR12	24 F D	24 F C	16 L B	24 F D
HR-SR9-C	24 L C	HR-SR6	BC4-6	BC4-7	HR-SR30	BC4-9	BC4-9	24 L D
HR-SR21	BC4-22	Rialto	HR-SR17	HR-SR14	BC4-26	16 L B	24 F C	Guard

Figure 2.4.11: Morley (S) plot randomisation 2015. This trial is split into two sections with a row of Guard plots, to accommodate the field tramlines. There are three 6m^{2}plots of each of the main NILs selected for further study (Table 2.2.2). In addition to these there are two replicated of the SPRI recombinant lines (Table 2.3.2).

Row 1	Row 2	Row 3	Row 4	Row 5	Row 6	Row 7	Row 8	Row 9
HR-SR14	HR-SR21	BC4-26	BC4-11	HR-SR30	Spark	HR-SR14	16 L A	24 L D
HR-SR30	BC4-9	Rialto	16 F A	16 L B	16 F B	HR-SR17	BC4-9	16 F B
24 L D	24 F C	BC4-6	HR-SR10	HR-SR10	BC4-7	HR-SR21	24 F C	BC4-11
16 F B	HR-SR15	HR-SR12	16 L B	HR-SR9-C	24 F C	HR-SR6	BC4-4	24 F D
Guard	Guard	Guard	Guard	Guard	Guard	Guard	Guard	Guard
HR-SR6	HR-SR10-C	BC4-22	24 F D	HR-SR10-C	HR-SR15	HR-SR12	BC4-22	24 L C
HR-SR17	BC4-7	HR-SR9	BC4-26	BC4-4	BC4-6	BC4-11	16 L B	16 F A
HR-SR9-C	24 F D	Spark	24 L D	24 L C	BC4-22	16 L A	BC4-26	BC4-6
16 L A	24 L C	BC4-4	16 F A	Rialto	HR-SR9	BC4-9	BC4-7	Guard

Figure 2.4.12: Morley (Y) plot randomisation 2015. This trial was split into two sections with a row of guard plots, to accommodate the field tramlines. There were three 6m^{2}plots of each of the main NILs selected for further study (Table 2.2.2). In addition to these there were two replicated of the SPRI recombinant lines (Table 2.3.2).

Trial	Replicates	Lines	Slot 16
Hercules S 2014	2	Main 15 +HV	
Track S 2014	2	Main 15 +HV	
Morley S 2014	2	Main 15 +HV	
Teagasc S 2014	2	Main 15	
Syngenta S 2014	1	Main 15	
Morley S 2015	3x Main 2x Rec	Main 15, 10 SPRI Rec	
Track Y 2014	3x Main 4x Slot16	Main 15	BC4-4,BC4-6,BC4-22
Morley Y 2014	3x Main 4x Slot16	Main 15	16LB,24LD,24FD
RAGT 2014	3x Main 4x Slot16	Main 15	24LC.16FB
Limagrain 2014	3x Main 4x Slot16	Main 15	16FA,16LA,24FC
Morley Y 2015	3x Main 2x Rec	Main 15, 10 SPRI Rec	

Table 2.4.2: List of lines included in the field trials. HV stands for height variability lines.

2.5 Standard plant protocols

Seedling germination

Seeds were germinated prior to sowing to ensure that the plants developed correctly and to prevent seeds that fail to germinate from affecting replication. Seeds of the required lines were placed in a Petri dish lined with filter paper (Whatman 90 mm, Whatman International Ltd, Hadstone, UK). Water was added to the Petri dish until the filter paper was completely covered. The number of seeds did not exceed approximately 30 per dish, as more than this made separating seeds difficult. In addition seeds of different lines are be germinated in different dishes to prevent mixing up different lines. When the Petri dishes were labelled and prepared, they were covered in foil to exclude light and kept in a cold room at 5°C. Two days later these dishes were transferred to a controlled environment cabinet where they are kept at 18°C. The range of time seeds can be left before use is wide but after another 2-3 days seeds were showing visible signs of germination. Depending on the timing of the set up of the rest of the experiment, they were potted up after 4-8 days.

Potting up plants

After germination seeds were grown as seedlings in trays of 60 or 96 wells. These trays allowed for a lot of seedlings to develop in a small space. However the small size of the individual sections will result in low tillering and unhealthy plants if the plants were kept in these trays until maturity. Once the seedlings reach approximately growth stage GS11 they were transferred to larger pots. For plastic glasshouse and glasshouse experiments 1 litre square pots (FP7's) were used. The 1 litre of soil provides plenty of space for the roots to develop in, and the dimensions of these square pots allowed for growing the plants close together in tessellated blocks. This was important for my experiments as some of them involved interplant interactions that required them to be grown close together.

2.6 Standard fungal protocols

Fungal isolate preparation

Work with fungal isolates was performed using aseptic techniques. Surfaces and implements were sterilized before use and between isolates and transfer of material was performed under a laminar flow hood. To grow fungal isolates for experimental use, potato dextrose agar (PDA) plates were made to contain the growing fungus. When these were set, Eppendorf tubes containing the desired isolates of *Z. tritici* were collected from storage in the -80°C freezer. Sterile cotton buds were then used to extract the desired isolates. The newly infected cotton bud was streaked onto a sterile PDA plate ensuring the whole plate was covered. This process was then repeated on a 2nd PDA plate as a back-up or on additional plates in situations where a lot of the isolate was required. Inoculated plates were sealed, labelled and placed into a growing cabinet (18°C) and left to develop for approximately 5 – 7 days.

Bulking Fungal isolates

To generate large amounts of *Z.tritici* for infection of larger areas, the standard fungal isolate preparation technique was insufficient. To create larger quantities the fungus was grown in Potato Dextrose Broth (PDB) for several weeks. Conical flasks of PDB were placed under a sterile laminar flow hood with a Bunsen burner to reduce the likelihood of other organisms contaminating the media. To further reduce unwanted growth, 20µl of a penicillin / streptomycin mix was added as an antibiotic. An isolate being bulked was added to each flask with an inoculating loop. This was sterilised with ethanol and a flame prior to use and between each isolate. When each flask has had the antibiotic and the fungus added, they were sealed with a bung and tinfoil at the top. Due to the possibility of contamination and the large amount of fungus desired, each isolate bulked up in at least 3 flasks. When all the flasks were prepared and sealed, they were kept in a shaker at 18°C at 150 rpm. To check how much fungus had grown, flasks were taken out of the shaker for an hour or two, allowing the fungus to settle to the bottom as a distinct layer. To allow enough of the fungus to grow to infect a small trial, the flasks were left for three to four weeks.

Spraying with a backpack sprayer

A 20 Litre backpack sprayer was used for inoculating disease trials and for adding nutrients or fungicides. Prior to use it was cleaned out by filling it with water and spraying a small region of ground to a) remove any traces of what it was last used for and b) to check that the nozzles are providing an even spray. Spraying was performed in conditions of cool to moderate temperature to prevent scalding and in low wind to prevent drift. The process started by adding the solution being sprayed to the tank, which was then strapped to the back of the operator. Spraying was performed at a height at which the liquid covered the plant material evenly. The spray operator walked at a steady pace to ensure that the rate of application was as constant as possible. Multiple passes over the same area were performed until the required volume had been sprayed. After infection, plants were covered with black plastic sheeting and given extra irrigation to raise humidity. This was removed after 48hrs and normal watering resumed.

Scoring Septoria tritici blotch (STB)

Septoria tritici blotch is a foliar disease that causes lesions to form on the leaves of wheat. These lesions are typically light brown in colour and occur in irregular shapes. The disease can be distinguished from other necrotic wheat diseases by the presence of small black pycnidia within the lesions.

The most commonly used method for measuring STB is involves the scoring the percentage of leaf area containing the black pycnidia (Stewart and McDonald, 2014, Kema et al., 1996a). This method was used for this project. The scale used is typical for disease assessment and assigns each leaf one of the following scores, spaced evenly on a logit transformed percentage scale. 0, 1, 2, 5, 7, 10, 15, 20, 30, 40, 50, 60, 70, 80, 85, 90, 93, 95, 98, 99, and 100%. As this scoring method required human judgement, scoring requires being trained to identify the symptoms and logging who performed the scoring to include as a factor. While the performance of a scorer may vary through a day, this factor is confounded by that of the blocks, which were scored in sequence.

After scoring had been performed on multiple time points, it was converted into an AUDPC score (area under disease progress curve) (Shaner and Finney, 1977). This was calculated by adding together the results of multiplying the average of each two time points by the time difference between them. This was converted to % AUDPC by comparing the data to the maximum possible AUDPC, which is the ADUCP that would have occurred if every scored value had been 100% (Figure 2.6.1).

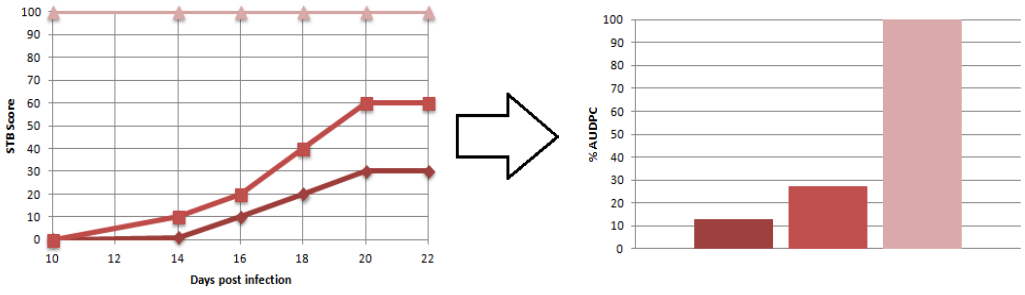

Figure 2.6.1: Conversion of STB scores over time to % AUDPC. Values used in this graph are not from any experiment and for illustrative purposes only.

2.7 Molecular techniques

DNA Extraction

Extraction of DNA for genotyping the lines was performed according to the following protocol. Extraction was performed on plates of 96 samples at once to allow the genotyping of many lines in a short time period.

Samples were collected by cutting 20-30mm sections from the leaves of plant being processed. These leaf samples were then placed in the wells of a 2ml 96 well collection plate, with 500µL of extraction buffer in each well. Extraction buffer was made using 100ml Tris-HCL, 100ml 0.5M EDTA and 125ml of 10% SDS in each Litre. Cell lysis was performed by disrupting the samples by shaking them for 3 minutes in a Genogrinder with 3mm ball bearings in each well. Afterwards the plates were incubated for 1hr at 65°C before being returned to room temperature. 250µL of 6M ammonium acetate was then added to each well, to precipitate proteins out of the solution. The plates were vortexed at a low speed to mix the solution and left for 15 minutes for the reaction to occur, prior to the plates being centrifuged at 4200rpm for 15 minutes to separate the debris. The DNA remained in the supernatant which was transferred into new plates containing 360µL iso-propanol. As the DNA is insoluble in this alcohol, this procedure aided the aggregation of the DNA into a pellet, when the plates were centrifuged again (4200rpm for 15 minutes). After centrifuging, the supernatant was removed leaving a pellet of DNA in the base of each well. The DNA samples were further processed by washing the pellet in 500 µL of 70% ethanol and centrifuging the plates a final time (4200rpm for 15 minutes). The supernatant was tipped out of the wells leaving the pelleted DNA ready for suspension in 200µL 1xTE buffer. The Tris-HCL and EDTA in the buffer help keep the DNA stable. The quality of the DNA extraction was then assessed by quantifying the DNA using a nanodrop (Nanodrop 2000, Thermoscientific).

Kaspar genotyping

After initial experiments using the SSR marker Psp3071, the majority of genotyping was performed using Kaspar The genotyping procedure used was adapted from the use previously described in (Trick et al., 2012). Primers have target SNP in the 3' end and either the FAM or VIC sequence at the 5' end (FAM GAAGGTGACCAAGTTCATGCT; VIC GAAGGTCGGAGTCAACGGATT). Every plate tested had six to twenty four samples of the parental lines included per marker. These were used to test if a) The SNP was differentiating between the parental lines, and b) To determine which parent the NIL lines aligned with for that marker. An example of reading the Kluster caller is given in Figure 2.7.1.

To perform Kaspar genotyping working stock primers were required for each marker being tested. Each working stock mix contained, 12µL of 100µM FAM primer, 12µL of 100µM VIC primer, 30µL of 100µL COM primer in 100µL. The primers were designed with the help of Ricardo Ramirez-Gonzalez and ordered from Sigma Aldrich. 2.5µL of DNA was added to each well of a 384 well sample plate (Cat. No. 04729749001, Roche Diagnostics). To replicate the results each source of DNA was included in multiple wells. Each well then had 2.5 µL of Kaspar master mix and 0.07 µL working stock primer added. Multiple wells were tested with the same marker, so a larger amount of Kaspar mix and primer would be combined and added to the wells with a multichannel pipette. Plates were then sealed with adhesive sealing sheet (Thermo scientific AB-0558) and run in mastercycler (Eppendorf pro384). The following program was used

- Hot-start at 94°C for 15 minutes
- 10 cycles of 94°C for 20 seconds then 65 °C for 1 minute
- 30 cycles of 94°C for 20 seconds then 57 °C for 1 minute
- Idle at 10°C

After the cycles were complete the florescence data was read in a Safire plate reader. If samples were not sufficiently amplified, they were run for an additional 5 or 10 cycles. The data was then analysed in Kluster caller by comparing the relative florescence of the FAM and VIC to the parental lines.

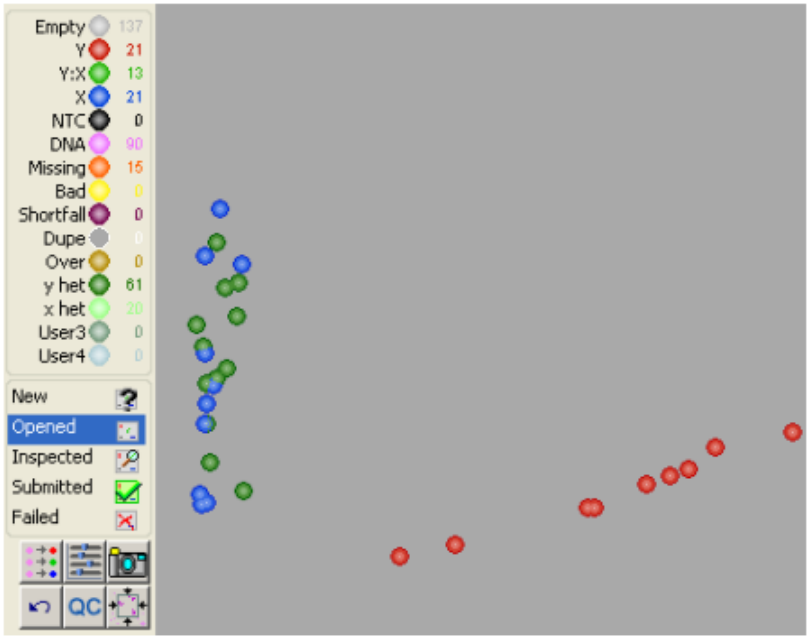

Figure 2.7.1: Example of Kaspar data in Kluster caller. Red dots represent the Flame parent, Blue dots represent the Longbow parent and Green dots are DNA samples from the 24A lines which were all Longbow for this marker, 6A_13/ BS0003881.

2.8 Statistical analysis

The experiments performed in this thesis were analysed using the statistical program Genstat 14th Edition (VSN International Ltd). The statistical models used for the experiments are given alongside the data in the relevant results chapters. The general linear model was used when the data was normally distributed, but for the disease data this often was not the case. When the data was not normally distributed the data was adjusted by the use of the logit function. Logit (X) = LN(X+F/(100-F-X)) where X is the value being transformed and F is half of the smallest possible value for X, to prevent undefined values of the logit function when X = 0% or 100%. This is noted alongside the statistical analysis where it was used. For experiments with multiple measurements within the unit of experimentation, e.g. multiple tillers per plant, multiple plants per plot, the analysis used the variance of the unit of experimentation in calculating statistical significance. This is also indicated on the experiments it was appropriate for.

3 Resistance of wheat leaves to *Zymoseptoria tritici* infection in relation to chromosome 6A genotype

3.1 Introduction

Our region of interest was identified as part of an association genetics study into field levels of Septoria tritici blotch (STB) (Arraiano and Brown, 2016). Analysis of the disease scores from these trials identified six QTLs that had highly significant relationship with STB levels. ($P<0.001$) The QTL on chromosome 6A, associated with the SSR/microsatellite marker psp3071, was selected for further study. This region on 6A was chosen as it explained the most variation in disease levels in the trials, 26.7% out of the 62% explained by all of the identified regions (Arraiano and Brown, 2016). A potential explanation for the large effect caused by the QTL is that there is a gene encoding resistance to STB within this region. This chapter tests if genetic variation in the NILs affects resistance to *Z. tritici.*

For the initial association genetics study (Arraiano and Brown, 2016) the field trials were exposed to the natural populations of *Z. tritici* as opposed to being inoculated with specific isolates. However, for this chapter, inoculated experiments were used to look for the presence or absence of resistance at the 6A locus. Both major gene and race non specific resistance can be identified using inoculation experiments. Detached seedling inoculation tests (Arraiano et al., 2001) and attached leaf seedling tests (Brading et al., 2002), have previously been used in the identification of the majority of *Stb* genes. For example, the identification of *Stb9* (Chartrain et al., 2009) used both types of seedling test to characterise the STB response.

Inoculated experiments using specifically cultured isolates add a greater amount of control and reliability to the fungal side of the interaction. This results in any differences in disease levels being more likely to be caused by the plant's defence pathways than external factors. Using specific isolates also facilitates testing if there is a variety specific component of resistance.

Seedling tests are useful in attempting to discover resistance genes as they can be performed with smaller amounts of seeds than field trials. The two different types also

have their own advantages. Attached seedling tests allow large populations to be screened for their responses to the pathogen quickly, whereas detached leaf tests are better at studying multiple isolates. This makes them a useful tool for identifying race non specific resistance, as to identify race non specific resistances, plant material needs to be inoculated with a wide range of isolates (Johnson, 1984). Identification of race non specific resistance occurs by identifying a significant reduction in disease levels compared to controls, which occurs independently of the isolate being used.

Seedling pathology tests can be strongly correlated with adult plant field data (Arraiano et al., 2001). However testing of adult plants in addition to seedling experiments is important as adult-plant responses to *Z. tritici* do not always reflect responses of seedlings to the pathogen (Kema and van Silfhout, 1997, Chartrain et al., 2004b). Stb17 has a quantitative effect on disease which is absent in seedlings but present in adult plants (Tabib Ghaffary et al., 2012) and genes on 5B have been linked to increased susceptibility only in adult plants (Arraiano et al., 2007). Wheat has also been shown to have yellow rust (Johnson and Taylor, 1972) and powdery mildew (Hague and Brown, 1996) resistance that only occurs in adult plants. Confirming that the disease response on the mature leaf tissue aligns with the seedling data is especially important because the flag leaf and second leaf are important for grain filling (Sanchez-Bragado et al., 2014, Khaliq et al., 2008) and loss of green leaf area caused by STB infecting the flag leaf has an impact on yield (Shaw and Royle, 1989a).

The QTL on 6A found in Arraiano and Brown (2016) has been bred in Flame x Longbow and Spark x Rialto NIL populations for further study (Chapter 2.2 & 2.3). These populations have not been previously studied in their response to pathogens. In this chapter, the 6A NILs response to inoculation is characterised in both seedlings and adult plants. The key questions to answer in this chapter are

1) How do the 6A NILs respond to inoculation with *Zymoseptoria tritici* ?
2) Is this response in seedlings the same in adult plants?
3) Does the QTL respond similarly in the different backgrounds?
4) Do inoculation tests support the hypothesis of the difference in predicted field levels being caused by a novel resistance gene?

3.2 Materials and methods

Materials:

The lines of wheat used in these experiments were the Flame x Longbow NILs and Spark x Rialto NILs (Chapter 2.2 & 2.3). Maris Pinion was used as the susceptible control in all experiments, whereas different resistant lines were used depending on the selected isolates (Table 3.2.1). The fungal isolates used are part of the Brown lab's collection of *Z. tritici*. Those selected for resistance tests are listed in the table below (Table 3.2.1). The earliest seedling tests were performed prior to genotyping and were genotyped from their DNA afterwards, the later tests used homozygotes from the genotyping of the G53 population (Chapter 2.2/Figure 8.1) The lines selected and plot layout of the adult plant tests were different for AP1 (Figure 2.4.1) and AP2 (Figure 2.4.2.). AP1 was performed in 2013 and used the BC2 SPRI lines, and four FLLO lines. AP2 was run after the selection of the main 15 lines (Figure 2.2.2), and also includes the SPRI recombinants in one block (Figure 2.3.2).

Isolate	Location of origin	Resistant line	Experiments
IPO 89011	Netherlands	Tonic	ST2,3,5,8
IPO 92006	Portugal	Bastard II	ST1,2,3
IPO 90012	Mexico	Heines 110	ST3,4
JIC040	Norfolk	*	ST6,7, AP1,2
ISR 398	Israel	Stb12 line**	ST5,8
IPO 94269	Netherlands	Stb10 line**	ST9, AP1
CHC3	Cheshire	*	ST7,9 AP1,2
CPC1	Northumberland	*	ST7
GR11	Gwent	*	ST7

Table 3.2.1: *Zymoseptoria tritici* isolates and resistant what genotypes used for inoculation experiments. * = Virulent to all known *Stb* genes. ** =These lines had been previously developed in the Brown lab to contain only the resistance genes Stb12 and Stb10 respectively.

Methods: Seedling experiment protocol (Attached)

Assessment of FLLO and SPRI NILs was done in a method adapted from Brading et al, (2002). Seeds were germinated (Chapter 2.5) then planted in 6x10 insert seedling trays containing John Innes Compost No 2 (1 seed per insert). Separate trays were used for each isolate included in the experiment. Seedlings were then grown in a controlled environment room. (16hr photoperiod, 70% humidity at 18°C and dark period at 12°C)

The seedlings were infected after growing for 12 to 16 days. Prior to this the appropriate fungal isolates were grown according to the standard fungal isolate preparation method (Chapter2.6). These were prepared seven days before infection to allow enough fungus to have grown on the plates. To prepare the isolates for inoculation, infected PDA plates were opened under a sterile flow hood and 10ml of distilled water was pipetted onto the plates. The fungus was then scraped into solution by a sterilised glass rod. The resulting fungal spore solution was then transferred into a falcon tube with a 10ml pipette. Any remaining visible fungus on the plate was collected via repeating the process to add more spores to the solution.

Infection of seedlings was done with a standard spore concentration of 10^7 spores per ml. Fungal spore solutions can have a wide range of concentrations based on the growth of the isolates being used. Thus they needed to be adjusted before use. The concentration of the conidial suspension was determined by diluting the solution hundredfold in an Eppendorf tube. A drop of diluted solution was added to a haemocytometer and pressed flat against it with a glass slide. The numbers of spores per haemocytometer square were counted under a light microscope several times. The following equations are applied to allow for the correct amount of the suspension to be diluted into an inoculation solution.

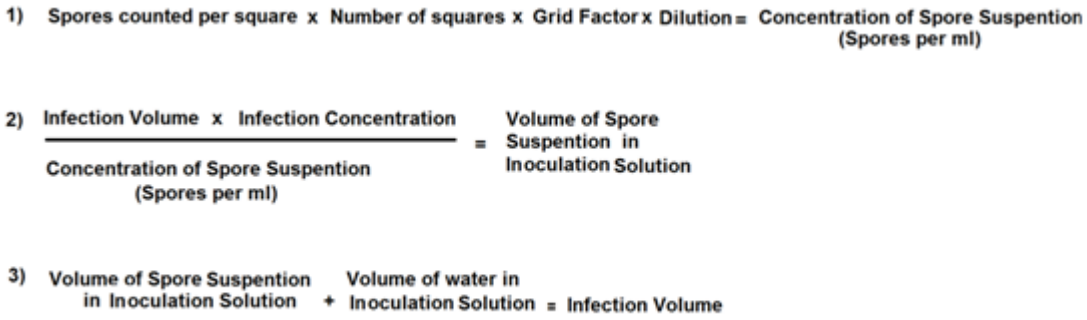

Figure 3.2.1: Spore Dilution Equations

The volume of inoculation solution made for each isolate was 100ml although only 25ml was used to inoculate each tray. The final stage of preparing the inoculation solution is the addition of a 25µl drop of polyoxyethylene-sorbitan monolaurate (Tween 20; Sigma-Aldrich Chemie, GmbH, Germany) to aid adhesion of the solution to the leaf surfaces. The inoculations were performed within the controlled environment room where the seedlings were being grown. To ensure even distribution with the inoculums the seedlings were rotated on a turntable (≈40rpm) during infection. Infections were performed using an air compressor (Clarke Wiz air, Clarke International) to distribute the inoculation solution over the rotating plants (Shaw, 1991). This ensures that the leaves were all visibly wet with inoculum by the end of the procedure. Between infections with different isolates all equipment was cleaned with 70% ethanol. To encourage successful infection the seedlings were then covered in a plastic lid and an opaque sheet for 48 hours. This led to low light and high humidity post infection which should increase stomatal opening providing more opportunities for the fungus to infect.

A problem with this method of infection is that the infected leaves can start to naturally senesce before they are showing symptoms. This was avoided by cutting back new growth as it appeared forcing the plant to prioritize its resources into the infected leaves keeping them alive for longer. This technique is especially important when working with *Z. tritici* because of its latent period of two to three weeks before symptom development. Scoring consisted of looking at the inoculated leaves of each seedling (either first leaf or both first and prophyll were scored) and determining percentage cover of infection due to *Z. tritici*. Scoring of STB by percentage was done

by visually estimating the amount of the leaf with lesions showing pycnidia. (Scale described in Chapter 2.6). Scoring was repeated every 2-4 days and was initiated prior to symptom emergence at around 14-16 dpi and then stopped at 30-40 dpi when leaves are sufficiently senesced to be not be capable of being scored accurately.

Seedling experiment protocol (Detached)

Seedling tests 1-3 were performed as detached leaf tests in a method adapted from Arraiano et al,(2001). This method differs from the previously described seedling test method in that after the inoculation of the plants, the inoculated leaves were cut in sections (30-50 mm) from the plant and kept suspended for scoring under a microscope.

Clear polystyrene boxes (120 x 80 x 20 mm) were used for suspending the leaf sections. These were filled with 50ml of 10g/L Agar-Agar and 100 mg/L Benzimidazole. Subsequently a template is used to remove the central region of the agar and the cut sections of the leaves are placed in the box facing upwards, bridging across the cut region. Then they were held in place by adding the agar strip from the cut region on top of the edges affixing them into position. As with the intact leaf tests these were then kept in the dark for 48hrs before being kept in a growth cabinet (20°C, white phosphorescent light (2x Philips TLD 70 W/83)) whilst the disease progresses.

AUDPC was calculated from the percentage of the cut leaf area covered by lesions bearing pycnidia. Scoring was performed after symptoms start appearing on the control susceptible lines and was repeated every 1-3 days until approximately 25-30 days after inoculation. All assessments were carried out using a binocular microscope.

Advantages of Detached	Disadvantages of Detached
Can be viewed in detail under microscope	**Increased chance of contamination due to greater handling**
Occupies less space	**Less like natural infection**
Scoring leaves is quicker	**Easy to manually damage leaves in setting up**
Easier to study multiple isolates	**Harder to screen large numbers**
Easier to randomise	

Table 3.2.2: Relative merits of detached leaf tests vs attached leaf tests for assessing levels of STB in seedlings.

Exp No	Type	Isolates	Plants per Allele (FLLO/SPRI)	DPI Range	No of time points	Average % STB	Leaf
ST1	Detached	IPO92006	50	17-35	6	23 %	1st
ST2	Detached	IPO92006 IPO89011	40 40	20-37	8	28%	1st
ST3	Detached	IPO90012 IPO92006 IPO89011	12 12 12	15-27	11	11%	1st
ST4	Seedling	IPO90012	60	22-35	4	8%	1st
ST5	Seedling	ISR398 ISR89011	46/35 46/35	20-35	6	17%	Prophyll 1st
ST6	Seedling	JIC040	60	21-42	6	15%	1st
ST7	Seedling	CHC3 CPC1 GR11 JIC040	24/22 24/22 24/22 24/22	17-30	4	13%	Prophyll 1st
ST8	Seedling	ISR398 IPO89011	48/20 48/20	22-34	4	12%	Prophyll 1st
ST9	Seedling	IPO94269 CHC3	48/36 48/36	20-33	4	11%	Prophyll 1st

Table 3.2.3: Experimental set up of the inoculated seedling tests (ST1-9). The plants grown per allele are listed for each isolate and separated into the lines used from the Flame x Longbow background and Spark x Rialto background. The number of timepoints and the range of days post infection (DPI) refer to when symptoms were observed on the leaves. These are variable due to the initial onset of symptoms, and leaves being too damaged to score, are dependent on the virulence of the isolate and success of inoculation.

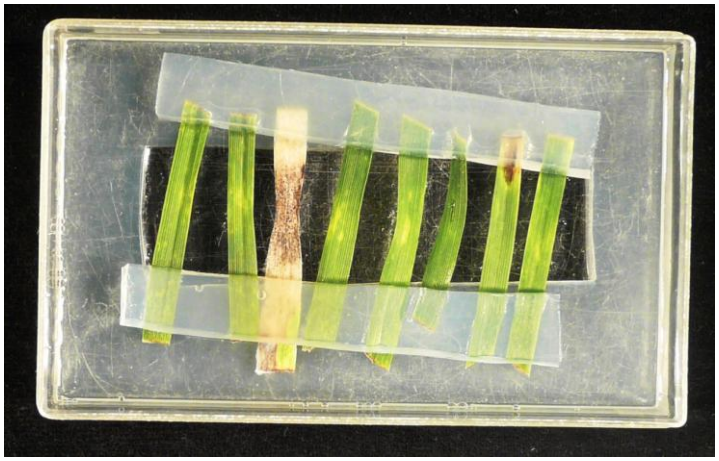

Figure 3.2.2: Example box from detached leaf test. The third leaf in from the left shows the susceptible control Maris Pinion.

Adult Plant experiment protocol

Exp No	Type	Isolates	Inoculation Date	Plants per Allele	DPI range	No of time points	Average % STB	Leaf
AP1	Adult: Glasshouse 2013	JIC040 IPO94269 CHC3	11th June 2013	32 32 32	22-43	4	21.15% AUDPC	Flag
AP2	Adult: Plastic glasshouse 2014	JIC040 CHC3	25th May 2014	64 64	17-31	4	47.81 % AUDPC	Flag

Table 3.2.4: Experimental set up of the inoculated adult plant tests (AP1-2). Each of the four alleles at the 6A locus (Flame, Longbow, Spark and Rialto) had the number of plants grown per allele for each isolate. The number of time points and the range of days post infection (DPI) refer to when symptoms were observed on the leaves

In the adult plant experiments, the seedlings were grown in 96 well trays until approximately growth stage 12 when they were potted up into FP7's. These 1ltr square pots are arranged in grids of 16 plants for each line (See plot layouts in Chapter 2.4). The plants were potted out in the winter to allow for natural vernalisation so that they were maturing similarly to field plants. This was done initially within an unheated greenhouse before being transferred to the environment where they would grow to maturity. Mildew was controlled during this time with treatment with Cyflamid.

Infecting large trials requires a bulked up fungus populations (Chapter 2.6). Conical flasks of the bulked isolates were then left for the fungus to settle to the bottom of the flasks. This allows the media to be removed with a 10ml pipette so that the fungus can be diluted in water without the rest of the medium. Spore concentrations were then adjusted with the equations shown in Figure 3.2.1. The major difference is that the volume required needs to be larger to cover the greater area and to prime the sprayer. Once the solution was prepared the trials were infected with a backpack sprayer. (Chapter 2.6)

The plants were being checked regularly as part of other experiments so as soon as visible symptoms appeared they started being scored. Scoring was performed on the

flag leaves with visual assessment of individual leaves as percentage of leaf showing lesions with pycnidia. (Chapter 2.6) Leaves were scored from all the tillers of the central four plants of the 16 plant plot. This provides a greater number of readings than just looking at the main stem of the plant and removes any edge effects. AP2 was both sown and inoculated earlier in the year than AP1 to allow scoring to be staggered with field trial scoring easier.

3.3 Results

Seedling experiments

Application of *Z. tritici* inoculum led to successful infection in all three detached leaf tests. There was some contamination with other pathogens which required the removal of the contaminated area. Analysing all three experiments together with experiment as a factor had greater statistical power and should provide a more accurate idea of the effect of the different alleles in the NILs. The statistical results of analysing the three experiments are shown in Table 3.3.1. The experiments are based around determining if the 6A locus affects the levels of STB. This is represented in the table by the significance of the factor Allele in the model. For the detached leaf tests the statistical analysis shows no significant difference for the influence of the alleles at the 6A QTL on STB.

ST1-3 Analysis of variance			
Variate: %_AUDPC			
Model: (Experiment*Isolate+Family)*Allele+Line			
	d.f.	v.r.	F pr.
Experiment	2	30.97	<.001
Isolate	2	1.42	0.24
Family	1	0.69	0.41
Allele	1	0.01	0.92
Experiment x Isolate	1	5.87	0.02
Experiment x Allele	2	0.12	0.89
Isolate x Allele	2	0.6	0.55
Family x Allele	1	3.45	0.06
Experiment x Isolate x Allele	1	0.07	0.79
Line	5	1.46	0.20
Residual	208		

Table 3.3.1: Analysis of variance for the level of STB in the three detached leaf tests (ST1-3). The STB was scored for multiple time points then converted to %AUDPC. Distribution of %AUDPC was sufficiently normal to not require transformation to logit %AUDPC. d.f refers to the degrees of freedom for the factor being added to the model. V.r is the variance ratio between the factor and the residual variance. F pr is a probability statistic based on the F distribution. A factor is considered to have a significant effect on the variate if the Fpr is less than 0.05.

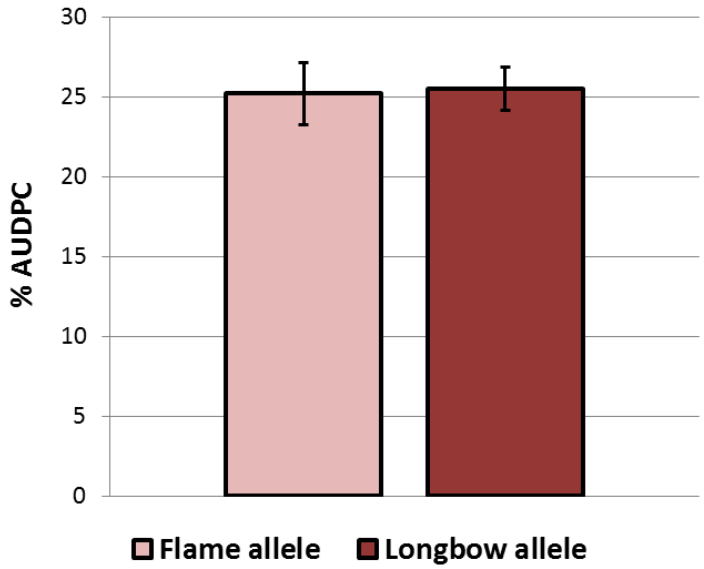

Figure 3.3.1: Predicted means of STB scores from the detached leaf seedling tests 1-3. The means were generated for the contrasting 6A alleles in the Flame x Longbow NILs using the model (Experiment * Isolate + Family)*Allele +Line. Error bars shown are +/- 1 standard error of the mean.

Figure 3.3.1 shows the predicted means for the two 6A alleles from the statistical model in Table 3.3.1. The response to infection by isolates of *Z. tritici* in the three detached leaf tests shows no significant difference after accounting for the other key factors. In Figure 3.3.2 this response is split into the individual isolates and experiments performed. Whilst there was a significant interaction between the isolates used and the experiment, neither of these traits significantly affected the effect of the 6A locus on the disease level.

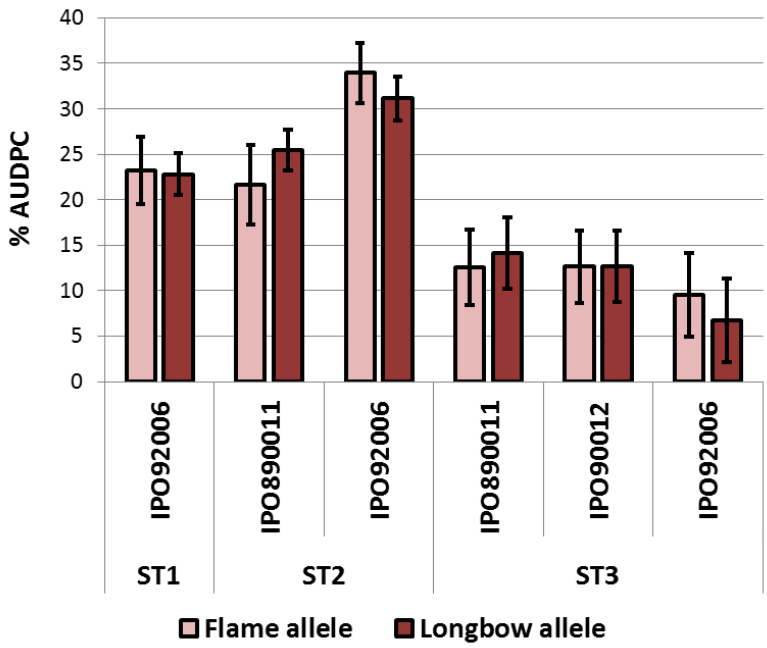

Figure 3.3.2: Predicted means of STB scores for isolates IPO92006, IPO89011 & IPO90012 in detached leaf seedling tests ST1, ST2 & ST3. The means were generated for the contrasting 6A alleles in the Flame x Longbow NILs using the model (Experiment * Isolate + Family)*Allele +Line. Error bars shown are +/- 1 standard error of the mean.

Seedling inoculation tests performed on attached leaves were also grouped together with experiment as a factor to aid analysis. These tests were performed on two different leaf layers so leaf is added as factor to the model**.** When the attached leaf experiments were being performed, Spark x Rialto seed was available for inclusion in testing. The SPRI data was analysed separately, so that the different population structures could be taken into account. The results of the Genstat analysis for the Flame x Longbow lines and Spark x Rialto lines are shown in Table 3.3.2 and 3.3.3 respectively. In accordance with the detached leaf tests, the key factor of Allele was not statistically significant in the Flame x Longbow background. This was also the case for the Spark x Rialto material, indicating that the 6A QTL didn't influence seedling STB levels.

ST4-9 Analysis of variance			
Variable: Logit % AUDPC (Flame x Longbow)			
Model: (Experiment+Isolate+Leaf+Family)*Allele+Line			
	d.f.	v.r.	F pr.
Experiment	5	33.97	<.001
Isolate	2	511.82	<.001
Leaf	1	40.27	<.001
Family	1	3.24	0.072
Allele	1	2.62	0.106
Experiment x Allele	5	5.39	<.001
Isolate x Allele	2	1.23	0.294
Leaf x Allele	1	0.62	0.433
Family x Allele	1	0.53	0.467
Line	60	3.2	<.001
Residual	773		

Table 3.3.2: Analysis of variance for the levels of STB on the leaves of the Flame x Longbow NILs in the attached leaf tests (ST4-9). Data transformed with the logit function to bring them to a normal distribution and to avoid undefined values for transformation of 0% and 100%. Logit (X) = LN(X+F/100-F-X) where X is the value being transformed and F is half of the smallest possible value for X. Abbreviations as in Table 3.3.1.

ST5-9 Analysis of Variance			
Variable: Logit %AUDPC (Spark x Rialto)			
Model: (Experiment+Isolate+Leaf+Family)*Allele+Line			
	d.f.	v.r.	F pr.
Experiment	3	17.7	<.001
Isolate	5	68.99	<.001
Leaf	1	37.31	<.001
Family	1	8.16	0.004
Allele	1	1.39	0.239
Experiment x Allele	3	0.19	0.906
Isolate x Allele	5	2.16	0.058
Leaf x Allele	1	2.4	0.122
Family x Allele	1	2.84	0.093
Line	5	1.18	0.318
Residual	438		

Table 3.3.3: Analysis of variance for the levels of STB on the leaves of the Spark x Rialto NILs in the attached leaf tests (ST5-9). Abbreviations are as in Table 3.3.1. Logit function as in Table 3.3.2.

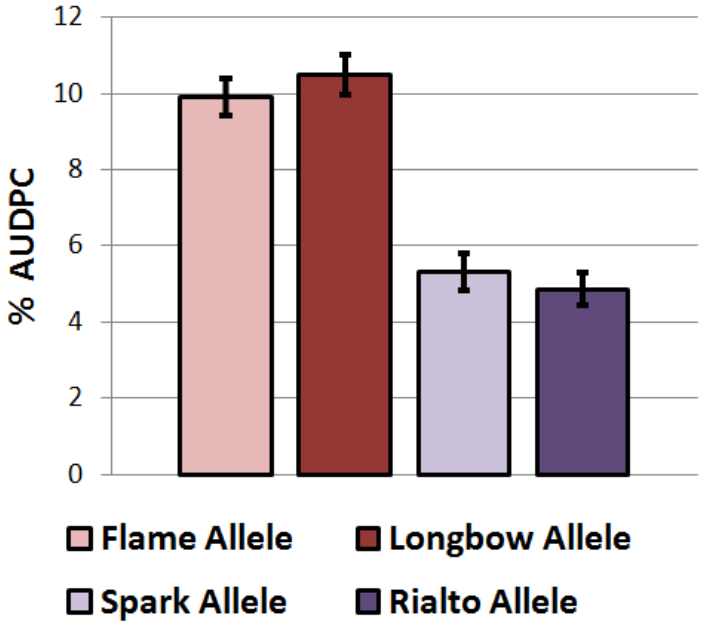

Figure 3.3.3: Predicted means of STB scores for the attached leaf seedling tests 4-9. The means were generated for the contrasting 6A alleles in the Flame x Longbow and Spark x Rialto NILs using the model (Experiment+ Isolate+ Leaf+ Family)*Allele+Line. Error bars shown are +/- 1 standard error of the mean.

There was no significant difference in the mean STB levels between the contrasting alleles at the 6A locus within the different populations (Figure 3.3.3). Dividing the data into individual experiments and isolate aids the interpretation of the statistics (Figure 3.3.4). The very large V.r values for isolate for the FLLO material can be attributed to ISR398 which showed very low levels of infection compared to the isolate IPO89011, which it was tested alongside in experiment ST5 and ST8. ISR 398 also led to low disease levels on the SPRI material as did the English isolates CHC 3 and CPC 1(Figure 3.3.5). Across all experiments (ST4-ST8) the NILs in each background produced similar %AUDPC values; with the exception of seedling test 9 where the Longbow allele was associated with significantly more symptoms than Flame. This response occurring in one out of the six experiments explains the Experiment x Allele interaction for the Flame x Longbow material (Table 3.3.2). No equivalent effect was seen in the Spark x Rialto material from the same experiment.

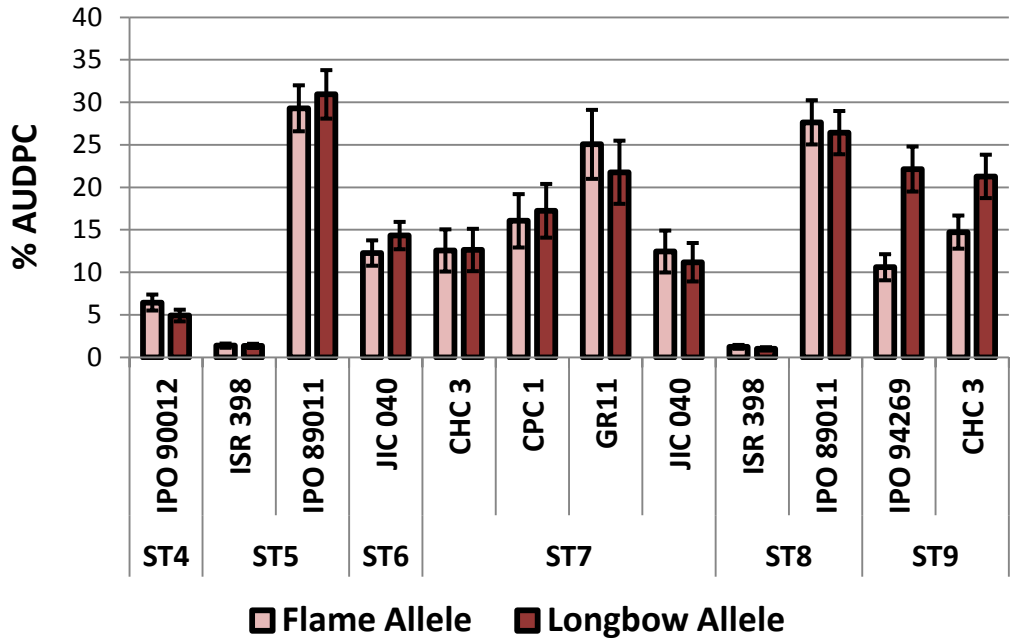

Figure 3.3.4: Predicted means of STB scores for each of the individual seedling test experiments. The means were generated for the contrasting 6A alleles in the Flame x Longbow NILs using the model (Experiment+Isolate+Leaf+Family)*Allele+Line. Error bars shown are +/- 1 standard error of the mean.

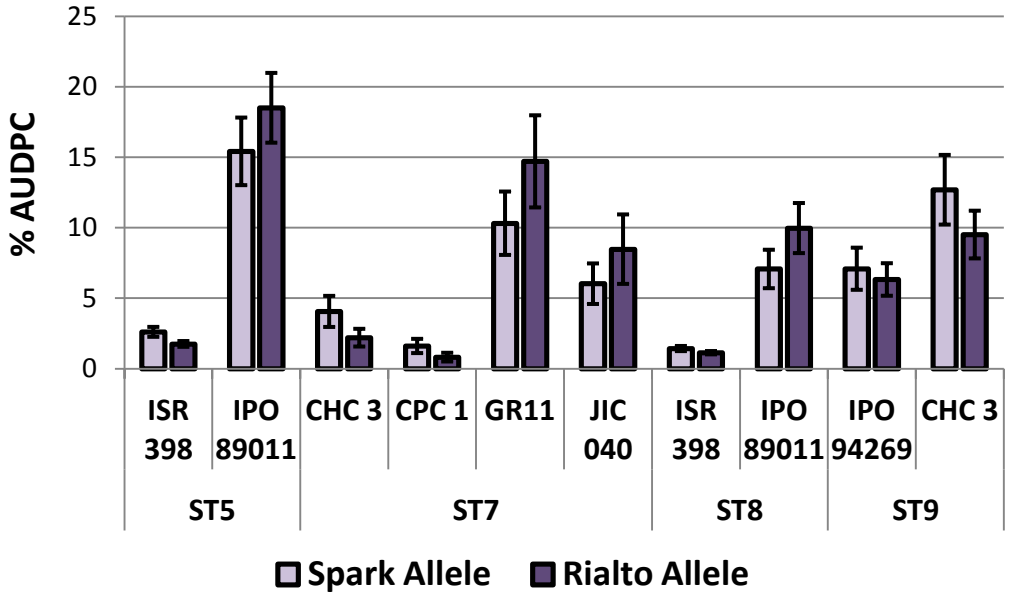

Figure 3.3.5: Predicted means of STB scores for different isolates and experiments in the seedling test experiments ST5-9. The means were generated for the contrasting 6A alleles in the Spark x Rialto NILs using the model (Experiment+Isolate+Leaf+Family) *Allele+Line. Error bars shown are +/- 1 standard error of the mean.

AP1 Results

The adult plant experiments AP1 and AP2 were analysed differently to the seedling experiments. Plots were arranged in two columns for each of the isolate tested and the factor of side tested to establish if this positional factor affected the STB data. It had no effect in either environment and was removed from the model. The FLLO analysis included family as a factor but all of the SPRI lines used were of the same family. For FLLO in AP1, there was only one line used for each family and allele combination making a line factor redundant. However, in SPRI "line" was included later in the model to account for the different lines with the same allele. Scoring in the adult plant tests was performed on all of the tillers of the four central plants of each block of 16. The plant position factor refers to which of four plants the reading was taken from, as plants in position 6 & 7 were further from the spraying apparatus (Chapter 2.6).

AP1 Analysis of variance			
Variable: % AUDPC (Flame x Longbow)			
Model: (Isolate+Family)*Allele+Plant Position			
	d.f.	v.r.	F pr.
Isolate	2	13.26	<.001
Family	1	13.42	<.001
Allele	1	0.06	0.812
0Isolate x Allele	2	0.15	0.864
Family x Allele	1	3.54	0.061
Plant Position	3	5.12	0.002
Residual	257		

Table 3.3.4: Analysis of Variance of % AUDPC of STB on the flag leaf for the Flame x Longbow NILs in AP1. Distribution of %AUDPC was sufficiently normal to not require transformation to logit %AUDPC. Abbreviations are as in Table 3.3.1

AP1 Analysis of Variance (SPRI ONLY)			
Variable: % AUDPC (Spark x Rialto data)			
Model: Isolate*Allele+Line+Plant Position			
	d.f.	v.r.	F pr.
Isolate	2	53.62	<.001
Allele	1	2.52	0.114
Isolate x Allele	2	3.95	0.02
Line	2	6.5	0.002
Plant_Position	3	4.02	0.008
Residual	290		

Table 3.3.5: Analysis of Variance of % AUDPC of STB on the flag leaf for the Spark x Rialto NILs in AP1 Distribution of %AUDPC was sufficiently normal to not require transformation to logit %AUDPC. Abbreviations are as in Table 3.3.1.

In both the Flame x Longbow and Spark x Rialto backgrounds, the factor of Allele did not show a significant effect on the levels of STB on the inoculated flag leaves (Table 3.3.4, 3.3.5 & Figure 3.3.8). The two different FLLO families, 16 and 24, affected the STB levels, as did the different isolates used. In the SPRI background the effect of the isolate interacted with 6A locus. Figure 3.3.9 shows that this interaction is significant due to NILs with the Spark allele at the 6A locus had higher levels of STB than the Rialto allele, when inoculated with IPO 94269.

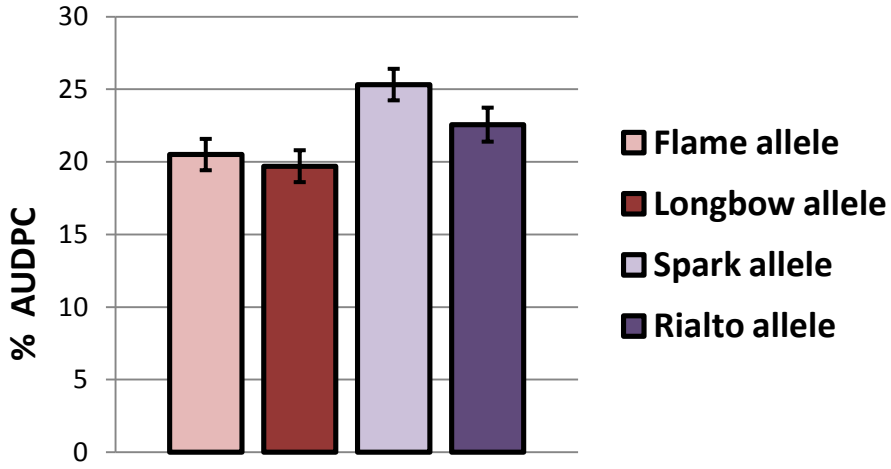

Figure 3.3.8: Predicted means of flag leaf STB scores in adult plant experiment 1 (AP1). The means were generated for the contrasting 6A alleles in both the Flame x Longbow and Spark x Rialto NILs. Error bars shown are +/- 1 standard error.

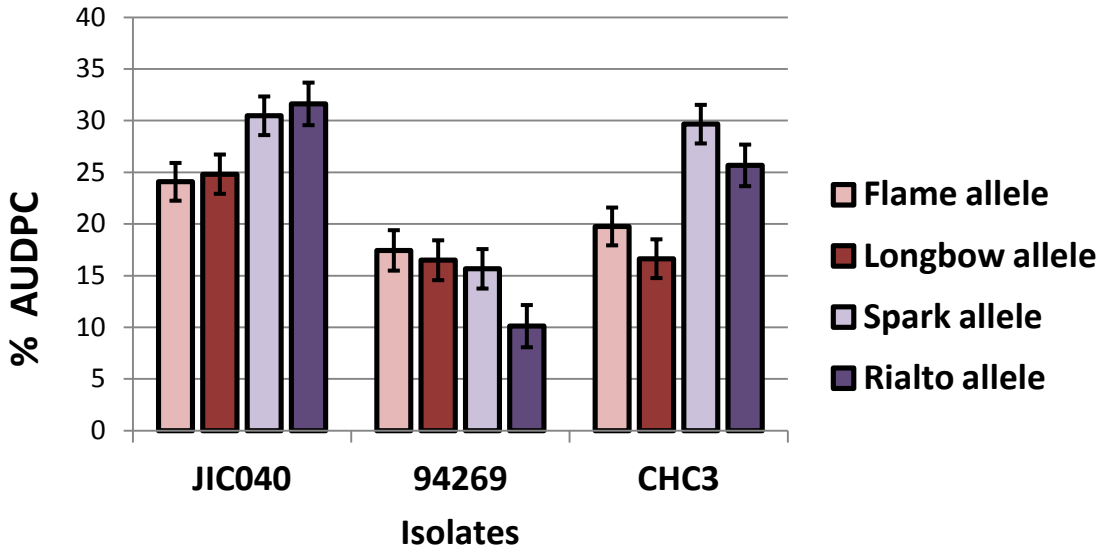

Figure 3.3.9: Predicted means of flag leaf STB scores for the different isolates in adult plant experiment 1 (AP1). The means were generated for the contrasting 6A alleles in both the Flame x Longbow and Spark x Rialto NILs. Error bars shown are +/- 1 standard error.

AP2 Results

AP2 Analysis of Variance			
Variable: % AUDPC (Flame x Longbow)			
Model: (Isolate+Family)*Allele+Line+Plant Position			
	d.f.	v.r.	F pr.
Isolate	1	3.12	0.08
Family	1	36.54	<.001
Allele	1	0.44	0.51
Isolate X Allele	1	2.86	0.09
Family X Allele	1	1.44	0.23
Line	4	4.31	0.002
Plant Position	3	0.36	0.97
Residual	182		

Table 3.3.6: Analysis of Variance of % AUDPC of STB on the flag leaf for the Flame x Longbow NILs in AP2. Distribution of %AUDPC was sufficiently normal to not require transformation to logit %AUDPC. Abbreviations are as in Table 3.3.1.

AP2 Analysis of Variance			
Variable: % AUDPC (Spark x Rialto)			
Model: (Isolate+Family)*Allele+Line+Plant Position			
	d.f.	v.r.	F pr.
Isolate	1	21.79	<.001
Family	1	19.49	<.001
Allele	1	1.43	0.234
Isolate x Allele	1	4.15	0.043
Family x Allele	1	2.34	0.128
Line	3	4.44	0.005
Plant Position	3	1.37	0.191
Residual	159		

Table 3.3.7: Analysis of Variance of % AUDPC of STB on the flag leaf for the Spark x Rialto NILs in AP2. Distribution of %AUDPC was sufficiently normal to not require transformation to logit %AUDPC. Abbreviations are as in Table 3.3.1.

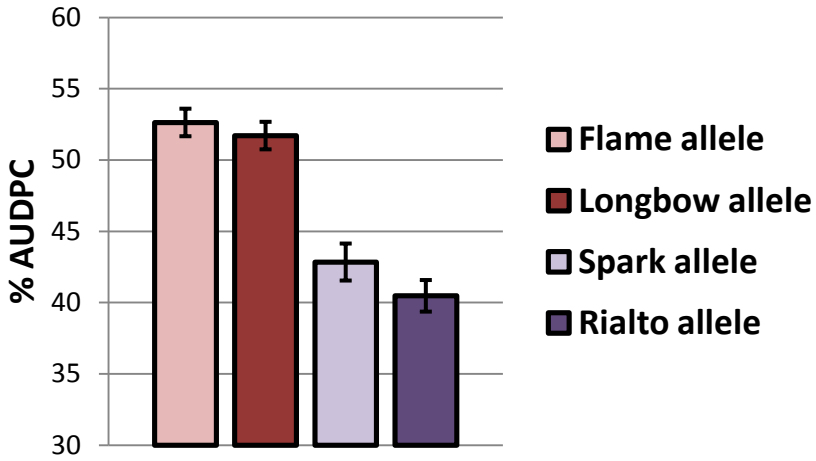

Figure 3.3.10 Predicted means of flag leaf STB scores for adult plant experiment 2 (AP2). The means were generated for the contrasting 6A alleles in both the Flame x Longbow and Spark x Rialto NILs. Error bars shown are +/- 1 standard error of the mean.

Tables 3.3.6 and 3.3.7 show the analysis of variance from analysing the flag leaf disease data collected from the Plastic glasshouse test in 2014. The different alleles at 6A didn't cause a significant difference in the levels of STB (Figure 3.3.10). The Flame x Longbow NILs in the 2014 adult plant STB trial had greater overall amounts of disease on their flag leaves, than the Spark x Rialto NILs (Figure 3.3.10). AP2 included more lines than AP1, with multiple lines for each family x allele combination, leading to the

inclusion of line as a factor in the model. Whilst line is significant in both backgrounds it has a relatively low variance ratio.

The different families within each background did lead to significant differences but these differences did not interact with the 6A locus**.** The most likely cause of variation between families is that different genes not linked to the 6A region were fixed in each family during the crossing programmes.

3.4 Discussion

The 6A QTL was predicted to have a large effect on levels of Septoria tritici blotch (STB) in the field. Inoculation experiments were performed, on near isogenic lines developed for the 6A region, to see if the field effects identified in the association genetics study could be observed in the NILs.

In most of the experiments performed for this chapter there were no significant differences between the NIL pairs. The only exception was in Flame x Longbow in Seedling test 9; however given the number of tests performed this can be attributed to a type 1 error. Susceptible controls of Maris Pinion consistently showed symptoms of STB in all seedling experiments indicating that inoculation had been performed correctly. Levels of disease varied between experiments and there were significant differences between the isolates used, though these can mainly be attributed to the different pathogenicity of the strains and variation in the conditions of the experiments.

The flag, prophyll and 1st leaves responded similarly to inoculation with *Z.tritici* in the adult plant experiments (AP1 & 2) and the seedling tests respectively. Both sets of experiments had no significant differences in STB attributable to the 6A alleles. The data from both sets of experiments are important, because whilst the primary infection in the field occurs on young plants in autumn, the yield loss from the disease is caused by the STB lesions reducing the photosynthetic capacity of leaves in the upper canopy (Robert et al., 2004, Shaw and Royle, 1989a).

Flame x Longbow NILs and Spark x Rialto NILs got different amounts of disease on average. This can be attributed to differing strengths or numbers of resistance genes in the rest of their genomes. As Flame and Longbow are more susceptible varieties they would be expected to get higher amounts of the disease as shown in AP2 and the attached leaf tests. That this was not the case in AP1 is unexpected. The two different families of Flame x Longbow had a different combination of Flame and Longbow material across the rest of their genome (Chapter 2.2). Family having a significant

effect in the adult plant tests indicates that this difference in the NILs background affected the resistance to STB. The difference between the two FLLO families (16 vs24) in adult plants did not occur in the seedling tests.

If there was a novel partial resistance gene in the region, there would be consistent variation in response to the pathogen, associated with the differing alleles at the locus. This difference could not be seen in the adult plant inoculation experiments and was also absent from the seedling inoculation experiments. Lack of evidence for the presence of a resistance gene is not the same as evidence for there being no resistance encoded there. However it adds legitimacy to considering alternative explanations for the field differences in disease.

The 6A QTL was predicted to cause differences in levels of field disease, but showed no significant differences in inoculated glasshouse trials. One of the main differences between the two types of experiment is how the fungal spores reach the flag leaves. In inoculated trials they are sprayed directly onto the scored region, but under conditions of natural infection, they arrive via splash borne transfer. This could lead to different levels of disease on the flag leaves if the physiology or anatomy of the plants was different between the NILs in a way that affected the spore transfer. The hypothesis that the NILs differ in traits related to disease escape will be tested in future chapters.

Summary

The purpose of this chapter was to characterise the response of the 6A NILs to flag leaf inoculation with *Z.tritici.* Based on the data from the association genetics study, the contrasting alleles at the 6A region were expected to differ in their response to the disease in the field. If this difference was due to R genes or susceptibility factors within the region, the inoculation experiments would to lead to worse symptoms of STB in the lines with the Longbow or Rialto alleles. This hypothesis was not supported by the experiments with Allele not being a significant factor in the analysis of the data. Whilst this does not definitively prove that there is no difference in the susceptibility of leaf tissue to infection by *Z.tritici*, it suggests it would be worth investigating the alternative hypothesis of disease escape causing the STB difference.

4 Candidate disease escape traits mapping to the 6A locus

4.1 Introduction

Tests on near isogenic lines for the 6A locus showed no significant differences in their response to inoculation with *Z.tritici* (Chapter 3). However this region may affect the levels of STB in the field via disease escape. Differences in developmental and physiological traits, including height and heading date, can alter disease escape (Arraiano et al., 2009, Simón et al., 2005, Van Beuningen and Kohli, 1990). If there are traits that affect spore transmission influenced by genes within the QTL, they could explain the predicted STB differences in the field caused by this region. In this Chapter a range of different traits are compared between NILs with the different alleles at the locus, to identify traits that may be involved in this process. A candidate trait would be one that is significantly affected by the different alleles at the NIL in both genotypes. In addition to this, the trait should influence disease escape. Crucially, to be a potential cause of the STB differences, the candidate trait should also operate in the same pattern as the projected disease differences. This pattern is that the Longbow and Rialto alleles would be affecting the trait in the same direction and that there would be a greater change in the trait between the FLLO alleles than in the SPRI background.

Plant height is an important escape trait to test because it affects disease escape in splash borne diseases like STB with a negative relationship between increased plant height and development of disease symptoms (Danon et al., 1982). In shorter plants, greater amounts of inoculum reach the upper canopy due to the shorter distance the spores need to spread. In addition to the overall height of the crop, the height of the individual leaves is also relevant. Because the disease moves up the plant one leaf at a time (Eyal, 1981), the effect of height can also be studies by looking at leaf spacing. Disease spread is reduced if there are larger gaps between leaf layers, as the spore transfer has to occur over a larger distance.

Heading date can affect the amount of disease in both foliar (Van Beuningen and Kohli, 1990) and ear diseases (Klahr et al., 2007). The effect of earlier plant development on disease levels is frequently explained as the pathogen having more time to cause damage in earlier developing plants(Van Beuningen and Kohli, 1990). A difference in heading date in the Spark x Rialto NILs has been discovered by Simmonds et al (2014), when studying grain development. They found that plants with the Rialto allele at 6A flowered 0.9 days earlier on average. Earlier heading date is often correlated with faster development at other growth stages. However, in the SPRI material the Rialto allele was associated with earlier heading but later overall plant maturity (Simmonds et al., 2014). As STB is a foliar disease the timing in leaf development is more likely to be directly related to differences in disease spread than the ear development. These differences in plant development associated with the 6A alleles, could contribute to disease escape in the lines. They also may not, heading date does not always show consistent association with disease levels, as indicated by it only having a minor effect that varied between trials on STB in Arraiano et al (2009) and it not affecting some diseases such as Ergot (Pageau et al., 1994).

Necrotrophic pathogens like *Z.tritici* induce cell degradation. Changes in the host's senescence responses may interact with the pathogen making it more susceptible to infection. If there was a different level of STB susceptibility between the NILs this would have been seen in the inoculation tests. However this does not rule out the possibility that difference in leaf development affect disease escape. Study of green canopy duration in the SPRI NILs found significant differences mapping to this locus (Simmonds et al., 2014). A different senescence profile or overall leaf lifetime may affect disease escape as they reduce the opportunity for spore transfer events.

Leaf area is a polygenic trait and is both a plausible candidate trait for disease escape but could also contribute to increased yield. Greater flag leaf area increases yield potential by providing a greater surface area for light interception and photosynthesis (Fischer and Kohn, 1966, Simpson, 1968). Increased leaf area may also affect disease escape by increasing the amount of plant tissue between which spores are transferred.

Leaf angle is controlled by multiple genes with additive effects (Dhindsa et al., 1992). More prostrate leaves have increased levels of spot blotch than those with an erect phenotype (Joshi and Chand, 2002) This is speculated to be due to changes in microclimate affecting success of infection rather than an alteration in spore movement. Prostrate leaves are associated with higher incidence of STB (Arraiano et al., 2009) although it has also been proposed that more erect leaves would increase spore transmission (Lovell et al., 1997).

Another factor that might be predicted to affect transmission of the disease is crop spacing. Greater crop densities increase the spread of another splash-borne pathogen, *Pyrenopeziza brassicae*, in oilseed rape (Pielaat et al., 2002). However Baccar et al (2011), found no strong differences in Septoria in wheat crops with different densities.

In addition to experiments to identify disease escape traits, experiments were also done to study the yield of the 6A NILs. This region of the genome carries genes that affect yield (Snape et al., 2007, Simmonds et al., 2014). In BC2 and BC4 SPRI lines, the QTL increases yield, TGW and grain width. In this chapter, yields are reported of lines with the Flame and Longbow alleles and in trials with high disease pressure.

4.2 Materials and methods

Environment	Flame x Longbow NILs	Spark x Rialto NILs	Blocking
2012 G63	180 lines sown, 94 homozygote lines identified.	NA	Plants randomized (No set blocks)
2012 G54	94 lines sown, 62 homozygote lines identified (Table 8.1)	NA	Plants randomized (No set blocks)
2013 G54	16A1, 16A2, 16A3, 16A5, 16B5, 16B8, 16B9, 24B7, 24B6, 24B18, 24C1 ,24C2, 24C4, 24C7, 24C16, 24D1, 24D2, 24D3 and 24D17.	NA	Plants randomized (No set blocks) 9 plants of each line
2013 G53	16A3, 16B8, 24C15,24D16	BC2-125, BC2-126, BC2-127, BC2-128	8 Blocks with 4 plants in each plot
2014 Plastic glasshouse	16A3, 16A4, 16B5, 16B13 24C15 24C16 24D1 24D16	BC4-4, BC4-6, BC4-7, BC4-9 BC4-11, BC4-22, BC4-26, HR-SR-9 HR-SR-9C, HR-SR-10, HR-SR-10C, HR-SR-15, HR-SR-21, HR-SR-12, HR-SR-14, HR-SR-17, HR-SR-6, HR-SR-30	2 Blocks of 16 plant plots SPRI recombinants only in 1 block
2014 Track Y	16A3, 16A4, 16B5, 16B13 24C15 24C16 24D1 24D16	BC4-4, BC4-6, BC4-7, BC4-9 BC4-11, BC4-22, BC4-26	3 Blocks of $6M^2$ field plots
2014 Morley Y	16A3, 16A4, 16B5, 16B13 24C15 24C16 24D1 24D16	BC4-4, BC4-6, BC4-7, BC4-9 BC4-11, BC4-22, BC4-26	3 Blocks of $6M^2$ field plots
2015 Plastic glasshouse	16A3, 16A4, 16B5, 16B13 24C15 24C16 24D1 24D16	BC4-4, BC4-6, BC4-7, BC4-9 BC4-11, BC4-22, BC4-26 HR-SR-9, HR-SR-9C, HR-SR-10, HR-SR-10C, HR-SR-15, HR-SR-21, HR-SR-12, HR-SR-14, HR-SR-17, HR-SR-6, HR-SR-30	Blocks of 16 plant plots SPRI recombinants only in 1 block
2015 Morley Y	16A3, 16A4, 16B5, 16B13 24C15 24C16 24D1 24D16	BC4-4, BC4-6, BC4-7, BC4-9 BC4-11, BC4-22, BC4-26 HR-SR-9 HR-SR-9C, HR-SR-10, HR-SR-10C, HR-SR-15, HR-SR-21, HR-SR-12, HR-SR-14, HR-SR-17, HR-SR-6, HR-SR-30	Blocks of $6M^2$ field plots SPRI recombinants only in 2 blocks

Table 4.2.1: The lines used and plot structure for the experiments in Chapter 4. The full randomisations and information on the creation of the NILs were given in Chapter 2.

Plant height and leaf spacing

Plant height (mm) was measured from soil level on the tallest tiller of the plant being measured. When the plant was mature, height was measured to the base of the ear. Before ear emergence, height was measured to the highest leaf tissue, typically the tip of the most recently emerged leaf. The height of individual leaves was measured to their respective leaf ligules.

Height (mm) (Ear base)	Reading	Flame allele	Longbow allele	Spark allele	Rialto Allele
2012 63		20	65		
2012 54		18	22		
2013 53		37	35	34	39
2013 54		59	55		
2014 Track Y		60	60	55	65
2014 Morley Y		28	28	18	24
2014 PT		32	32	24	32
2015 Morley S	1st	120	120	140	110
2015 Morley S	2nd	120	120	140	110
2015 Morley Y		48	48	56	44
2015 PT		48	48	36	48
Total		**570**	**568**	**503**	**472**

Table 4.2.2: Number of height (mm) (Ear base) measurements taken in each experiment.

Height (mm) (Leaf Ligules)	Leaf	Flame allele	Longbow allele	Spark allele	Rialto allele
2012 63	1	20	65		
2012 54	1	18	22	12	12
	2	18	22	12	12
	3	18	22	12	12
	4	18	22	12	12
2013 53	1	56	54	50	54
	2	40	40	39	41
	3	40	40	37	38
	4	40	39	36	37
	5	40	38	34	35
2013 54	1	56	54	50	54
	2	40	40	39	41
	3	40	40	37	38
	4	40	39	36	37
	5	40	38	34	35
2014 Track Y	1	76	76	71	81
	2	76	76	71	81
	3	76	76	71	81
	4	16	16	16	16
2014 Morley Y	1	62	73	45	60
	2	62	73	45	60
	3	62	73	45	59
	4	36	41	26	32
2014 PT	1	48	48	36	48
	2	48	48	36	48
	3	48	47	36	48
	4	16	22	15	25
2015 Morley Y	1	132	132	121	154
	2	12	12	11	14
	3	12	12	11	14
	4	12	12	11	14
2015 PT	1	32	32	24	32
	2	32	32	24	32
	3	32	32	24	32
Total		**1414**	**1508**	**1179**	**1389**

Table 4.2.2: Number of height (mm) (Leaf ligule) measurements taken in each experiment.

Leaf Senescence

The percentage of the leaf that was senescent was scored by measuring the distance from the leaf tip to the point where 50% of the leaf width was green. This length is then compared to the total leaf length to calculate a percentage (% Senescence).

Another way of quantifying the degree of senescence is to use a SPAD (Soil Plant Analysis Development) meter (Wood et al., 1993). A SPAD meter is a hand held light meter used to measure the relative transmittance of the leaf between the two readings (600-700nm and 400-500nm). The ratio between the different light readings is given in the unit SPAD value, where 0.0 corresponds to translucent and 50.0 to a fully green leaf. The readings this comparison produces are proportional to the amount of the chlorophyll in the leaf.

To get an accurate representation of the senescence of the leaf, at each time point measured SPAD readings were taken at four evenly spaced points along the length of the leaf, all data analysed is the average of these four readings. Both SPAD and percent senescence readings were organised to have an initial reading taken before senescence started to set the baseline level of greenness in the material, then additional readings were taken once the leaves are visibly senescing. The number of readings and the number of samples are shown in Table 4.2.3. The 2013 experiments had more time points and also tested the fifth leaf and had more replication overall. Whereas due having to score alongside multiple other traits the later field trials had less replication.

SPAD average	Readings	Leaves	Flame allele	Longbow allele	Spark allele	Rialto allele
2012 54	195	1,2,3,4	48	48		
	228	1,2,3,4	72	88		
	236	1,2,3,4	44	52		
	239	1,2,3,4	72	88		
	252	1,2,3,4	72	88		
2013 53	149	1,2,3,4,5	80	80	70	75
	155	1,2,3,4,5	80	75	65	75
	168	1,2,3,4,5	80	75	75	80
	177	1,2,3,4,5	120	115	80	80
	190	1,2,3,4,5	115	125	70	75
	197	1,2,3,4,5	160	160	105	115
2013 54	141	1,2,3,4,5,	220	200		
	155	1,2,3,4,5,	140	130		
	175	1,2,3,4,5,	160	165		
	193	1,2,3,4,5,	160	165		
2014 Track Y	155	1,2,3,4	64	64	64	64
	185	1,2,3,4	176	168	132	168
2014 Morley Y	143	1,2,3,4	80	100	60	80
	170	1,2,3,4	64	80	48	64
	188	1,2,3,4	72	72	48	72
	199	1,2,3,4	72	108	72	96
2014 PT	152	1,2,3	48	48	36	48
	160	1,2,3	96	96	72	96
	167	1,2,3	96	96	72	96
2015 Morley Y	161	1,2,3,4	48	48	44	56
	167	1,2,3,4	48	48	44	56
2015 PT	148	1,2,3	48	48	36	48
	168	1,2,3	48	48	36	48
	182	1,2,3	48	48	36	48
Total			**2631**	**2726**	**1265**	**1540**

Table 4.2.3: Number of SPAD average measurements taken in each experiment. The readings column shows the date of the reading converted into single number. The number of leaves tested varied based on how diseased or senesced the leaf was when the readings began.

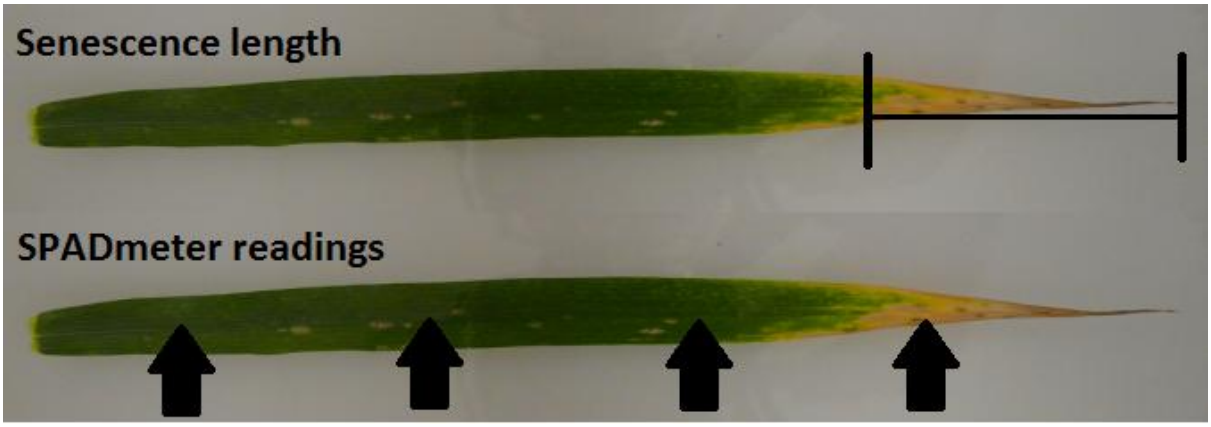

Figure 4.2.1: Image showing how percent senescence readings and SPAD meter readings would be taken on a typical leaf.

The senescence of lower leaves in seedlings was tested with the same method as the adult plants. This was tested in an additional experiment on 360 seedlings grown from the homozygote genotypes identified in Table 8.1. SPAD readings were taken on the prophyll and first leaf for 13 different time points.

To characterise the senescence response in more detail, six dark induced senescence tests were performed. Square boxes (235mm x 235mm) were filled with filter paper and 12 leaves were laid in parallel across the plate. Each box was divided into two sections to prevent leaves from different lines from being mixed up. Eight lines were included in these experiments, BC4-4, BC4-6, BC4-22, BC4-26, 16A3, 16A4, 24C15 and 24C16. The plates were stored in the dark in a cabinet set at 18°C. Every 2 days SPAD readings were taken on every leaf until 8-10 days when the leaves were no longer suitable for use in the SPADmeter. When these readings were being performed, additional water was added to keep the leaves from drying out.

Leaf Length (mm) and Leaf Area (mm^2)

Leaf length was measured from the ligule to the leaf tip to the nearest millimetre. In 2012, leaf width was also measured. In S63 this was done once across the centre of the leaf and in G54 every 60mm along the leaf. Total leaf area was estimated in G54 in 2012 by calculating the area each section as a trapezoid (Fig 4.2.3).

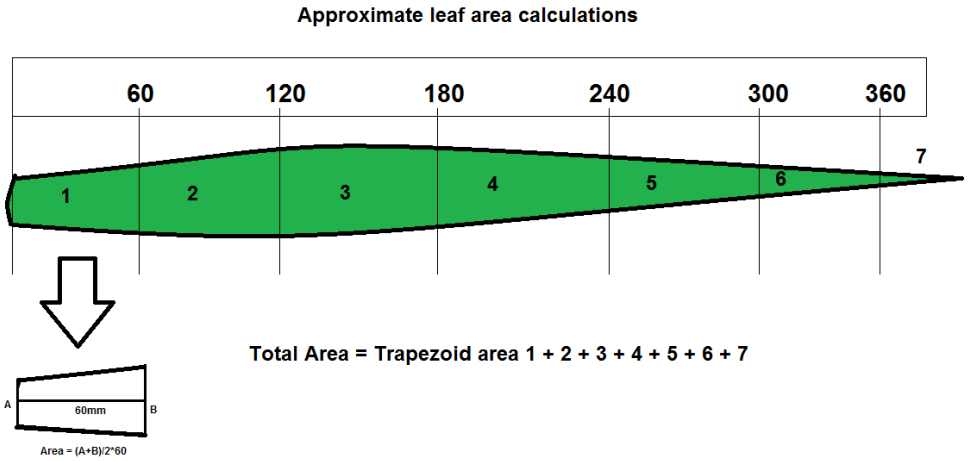

Figure 4.2.3: Pictorial representation of the calculated leaf area measurements in G54 in 2012

In G53 2013 and the subsequent plastic glasshouse and field experiments, there were sufficient plants to sample destructively. Sampled leaves were placed between two transparent plastic sheets on top of a light box and photographed from a distance of 42mm. This method minimised background variation and gives a clearly defined leaf edge. A script was written for ImageJ to convert batches of 120 images to black and white images measure the percentage of black pixels, and thus calculate leaf areas.

Depending on the extent of senescence when samples were collected, leaf area was measured on the top 3-4 leaves of the plant. In 2014 & 2015 when the majority of leaf area work was performed, this was done on the four largest tillers of the plant, and then the flag leaf was measured on every tiller. The extent of sampling is shown in Table 4.2.4.

Leaf Area	Leaf	Flame allele	Longbow allele	Spark allele	Rialto allele
2014 Track Y	1	45	63	68	67
	2	32	31	30	32
	3	32	32	31	32
	4	32	32	31	32
2014 Morley Y	1	21	24	22	26
	2	15	20	12	16
	3	14	20	12	14
	4	8	8	7	2
2014 Plastic glasshouse	1	32	18	36	31
	2	15	15	12	15
	3	13	13	12	12
2015 Morley Y	1	60	60	55	70
	2	60	60	55	70
	3	60	60	55	70
2015 Plastic glasshouse	1	41	41	38	52
	2	16	16	12	16
	3	16	16	12	16
Total		512	529	500	573

Table 4.2.4: Number of leaf area measurements taken in 2014 and 2015.

Other physiological and developmental traits

Ear length (mm)

Ear length (mm)	Flame allele	Longbow allele	Spark allele	Rialto allele
2012 63	20	65		
2012 54	18	22		
2013 54	60	55		
2014 Track Y	76	76	71	81
2014 Morley Y	42	48	30	40
2014 Plastic glasshouse	32	32	24	32
2015 Morley Y	48	48	44	56
2015 Plastic glasshouse	96	96	72	96
Total	392	442	241	305

Table 4.2.5: Number of ear length (mm) measurements Ear length (mm) was measured from the ear base to the ear tip..

Leaf angle

Leaf angle was scored by two methods, the first being to calculate the angle of the leaf by trigonometry after measuring the length of the leaf to the point of inflection and the distance between it and the stem. In later work, a visual assessment was used instead, using the following 9 point scale

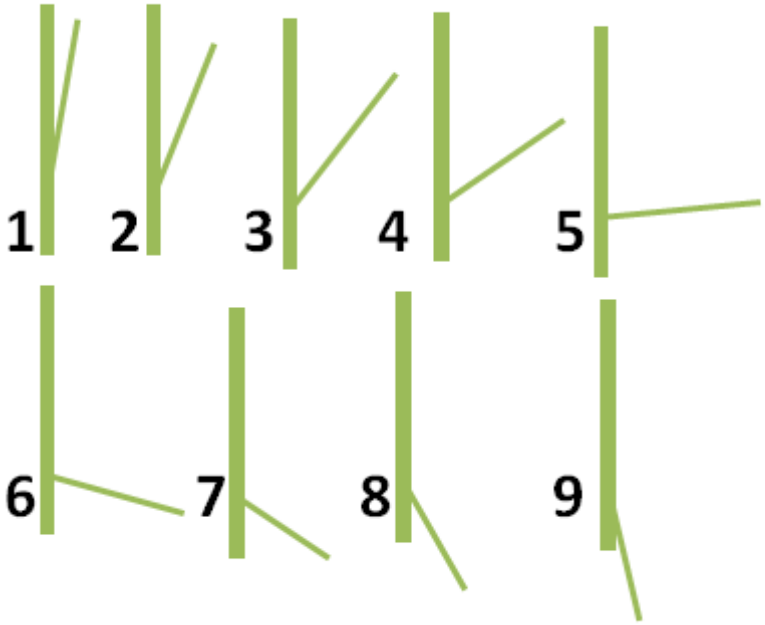

Figure 4.2.4: Leaf angle scoring guide on 1-9 scale.

Leaf angle	Flame allele	Longbow allele	Spark allele	Rialto allele
2012 63 (trig)	55	52		
2012 54	72	88	48	48
2014 Track Y	64	64	64	64
2014 Morley Y	64	76	47	59
2014 Plastic glasshouse	48	48	36	48
2015 Morley Y	48	48	44	56
2015 Plastic glasshouse	48	48	36	48
Total	399	424	275	323

Table 4.2.6: Number of leaf angle measurements

Growth stage

Plants were assessed for their Zadoks growth stage (Tottman and Makepeace, 1977) in early seedling experiments. In later work the approximate growth stage each plot was at when readings were taken was noted.

Heading date

In the field trials heading was scored as percentage of plants with ears emerged. In the glasshouses heading was measured as the developmental stage of tagged ears expressed as a percentage.

Leaf emergence

Leaf emergence in seedlings was measured by recording three traits on each leaf layer 1) whether or not any of the leaf was visible, 2) the length of the leaf and 3) whether or not the leaf ligule had formed. These readings were taken regularly on the on 360 (9 per line) seedlings grown from the homozygote genotypes identified in Table 8.1. This data set also recorded of the total number of leaves fully emerged. Readings were taken between when the plants were 22 and 65 days old. An additional test of leaf emergence was performed using the main 15 NILs in a controlled environment room (West 1) which also included SPRI NILs.

Ground coverage

Ground cover measurements were taken by photographing each 2014 field plot at 1.5 m above the ground on two occasions (28th March and 21st April). These photographs were then analysed using a macro developed by Oscar Gonzales which converts green pixels into black and all other colours to white.

Microclimate

Canopy temperature was measured by using a Raytek ranger ST to measure the temperature of the field plots. Two readings were taken for each plot, one of the temperature of the canopy and another of the soil temperature. This plot by plot measure was only performed twice to complement more regular climate data from local monitoring points.

Fresh weight (g) and Dry weight (g)

Whole plants were taken to the lab for measurements that could not be undertaken in the field. Leaves were weighed immediately after removal from the stem, on a laboratory balance (Mettier PT300). After the fresh weight reading had been taken the leaves were stored in bags and left to dry out completely in a drying cabinet, before being weighed again.

Fresh weight/ Dry weight	Flame allele	Longbow allele	Spark allele	Rialto allele
2014 Track Y	64	64	64	64
2014 Morley Y	64	76	47	59
2014 Plastic glasshouse	48	48	36	48
2015 Plastic glasshouse	48	48	36	48
Total	224	236	183	219

Table 4.2.6: Number of leaf weight (g) measurements.

Yield and 1000 Grain weight

Plot yields of the Norfolk trials were weighed during combining by scales built into the machine. Plot yields in trials run by collaborators were measured by them. The field trial plots had their 1000 grain weight calculated by a MARVIN grain analyser (www.gta-sensorik). The MARVIN scans 300-400 seeds, counting the number of seeds, and measuring the width, length and area of individual seeds, and weighing them to obtain 1000 GW. There was one 1000GW sample taken per plot, so the amount of sampling is the same as the replication in the experiment.

4.3 Results

Due to studying multiple traits in eight different main experiments, for the two different sets of genotypes, the analysis of variance tables for each trait in each experiment will not be presented. Instead the most important traits had data from all the experiments where they were tested pooled together to test the effect of the 6A NIL on each major trait. Minor physiological and developmental traits were only tested in one or two experiments and are summarised in Table 4.3.8.

The analysis of the following experiments was performed using variants of the following model: (**Year+Type+Location+Timepoint+Leaf+Family)*Allele+Line+Unit.** The factor **Type** in the model refers to whether the experiment was performed in a glasshouse/plastic glasshouse or in field conditions. The factor of **Unit** is used to generate the variance ratio used in the analysis. It is the plot number for the field trials and the plant number for the smaller experiments as these are the relevant units of experimentation. When analysing the experiments together, spatial factors such as the block in field trails and the plant position in the plastic glasshouse, are not included in the model, because the different experiments had different spatial structures. However, all experiments were also analysed individually with these factors included and they were usually insignificant.

Variate: Height (Ear Base)							
Model: (Year+Type+Location+Timepoint+Family)*Allele+Line+Unit							
Flame x Longbow NILs				**Spark x Rialto NILs**			
	d.f.	v.r.	F pr.		d.f.	v.r.	F pr.
Year	3	540.08	<.001	Year	2	48.42	<.001
Type	1	85.76	<.001	Type	1	83.39	<.001
Location	3	297.86	<.001	Location	1	31.60	<.001
Timepoint	6	34.56	<.001	Timepoint	3	6.67	<.001
Family	1	91.26	<.001	Family	2	1.09	0.36
Allele	1	2.04	0.15	Allele	1	2.69	0.10
Year x Allele	3	4.18	0.007	Year x Allele	2	0.48	0.62
Type x Allele	1	0.72	0.40	Type x Allele	1	0.86	0.35
Location x Allele	3	0.76	0.52	Location x Allele	1	0.84	0.36
Timepoint x Allele	6	0.73	0.63	Timepoint x Allele	3	0.44	0.72
Family x Allele	1	9.35	0.002	Family x Allele	1	0.24	0.63
Line	7	1.46	0.18	Line	5	0.68	0.64
Unit	279			Unit	107		
Residual	905			Residual	343		

Table: 4.3.1: Analysis of variance of the height to the base of the ear of the Flame x Longbow and Spark x Rialto NILs. d.f refers to the degrees of freedom for the factor being added to the model. V.r is the variance ratio between the factor and the residual variance. F pr is a probability statistic based on the F distribution. A factor is considered to have a significant effect on the variate if the Fpr is less than 0.05. The variance ratios were generated using the variance of the factor of unit instead of the residual.

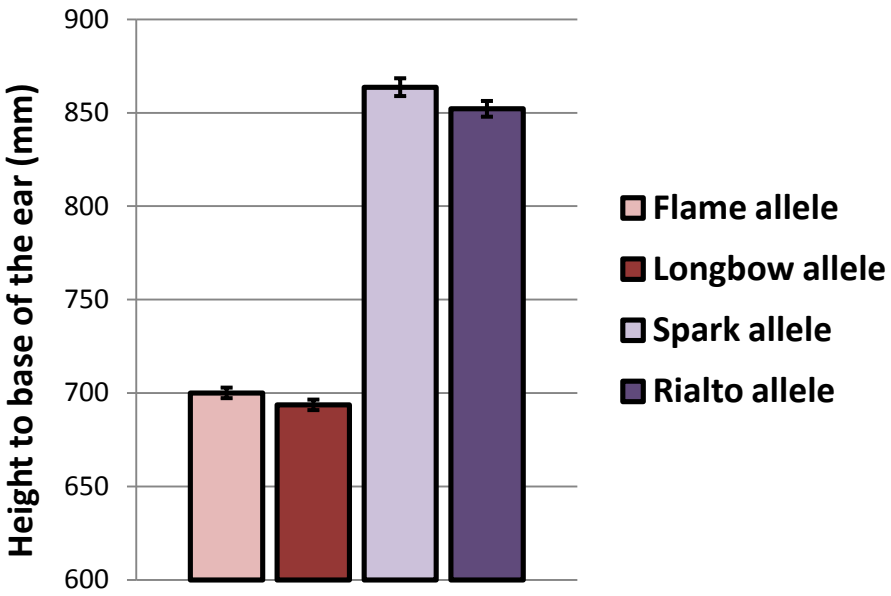

Figure 4.3.1: Predicted means for plant height to base of ear across all experiments where the trait was measured. The means were generated for the contrasting 6A alleles in the Flame x Longbow and Spark x Rialto NILs. Error bars are +/-1 standard error of the mean.

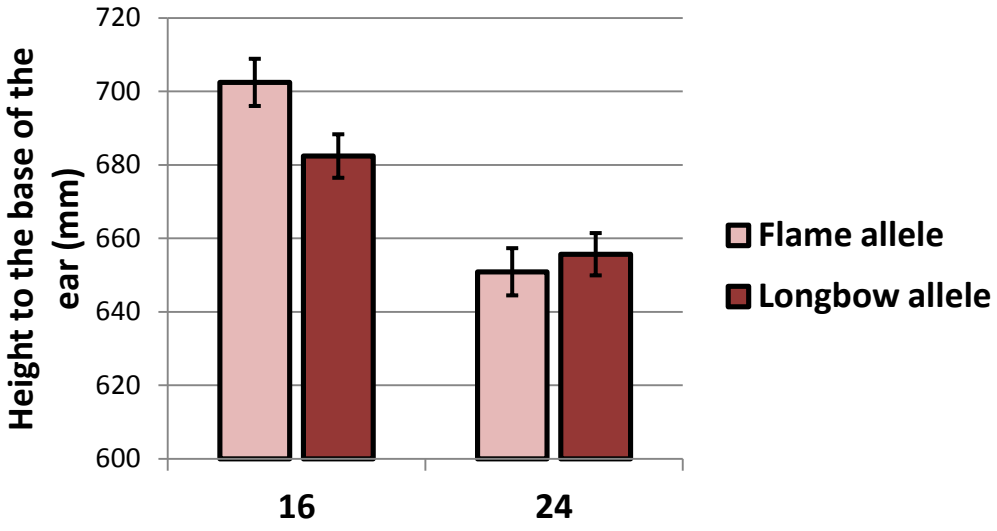

Figure 4.3.2: Average plant height to the base of the ear for the different Flame x Longbow families. The means were generated for the contrasting 6A alleles in the two different Flame x Longbow families. Data was pooled from all experiments where the trait was measured. Error bars are +/-1 standard error of the mean.

Across experiments there wasn't an overall significant difference in final plant height affected by the QTL on the 6A chromosome in either genetic background (Table 4.3.1). There was much variation in height between different experiments as shown by the high V.r values for year, type and location, though these did not interact with the effect of the allele. There was also a significant difference between the two families of FLLO with plants from family 16 being 30-50mm taller than plants from family 24. This difference interacts with the alleles at the 6A locus with the Flame allele increasing plant height in the 16 background but not 24 (Figure 4.3.2).

Variate: Height (Leaf ligules)							
Model: (Year+Type+Location+Timepoint+Leaf+Family)*Allele+Line+Unit							
Flame x Longbow NILs				**Spark x Rialto NILs**			
	d.f.	v.r	F pr.		d.f.	v.r.	F pr.
Year	3	1017.12	<.001	Year	2	283.49	<.001
Type	1	3.74	0.05	Type	1	13.95	<.001
Location	3	116.23	<.001	Location	1	126.2	<.001
Timepoint	12	66.05	<.001	Timepoint	10	83.24	<.001
Leaf	4	2474.30	<.001	Leaf	4	1580.62	<.001
Family	1	10.98	0.00	Family	3	1.38	0.25
Allele	1	0.92	0.34	Allele	1	0.22	0.64
Year x Allele	3	1.53	0.21	Year x Allele	2	0.88	0.41
Type x Allele	1	1.08	0.30	Type x Allele	1	7.15	0.008
Location x Allele	3	0.32	0.81	Location x Allele	1	0.53	0.47
Timepoint x Allele	12	1.25	0.25	Timepoint x Allele	10	0.67	0.75
Leaf x Allele	4	0.88	0.48	Leaf x Allele	4	0.069	0.99
Family x Allele	1	5.67	0.02	Family x Allele	1	4.85	0.029
Line	7	1.48	0.17	Line	5	0.30	0.91
Unit	290			Unit	188		

Table: 4.3.2: Analysis of variance of the height of the ligule of the individual leaf layers in Flame x Longbow and Spark x Rialto NILs. Abbreviations as described in Table 4.3.1. The variance ratios were generated using the variance of the factor of unit instead of the residual.

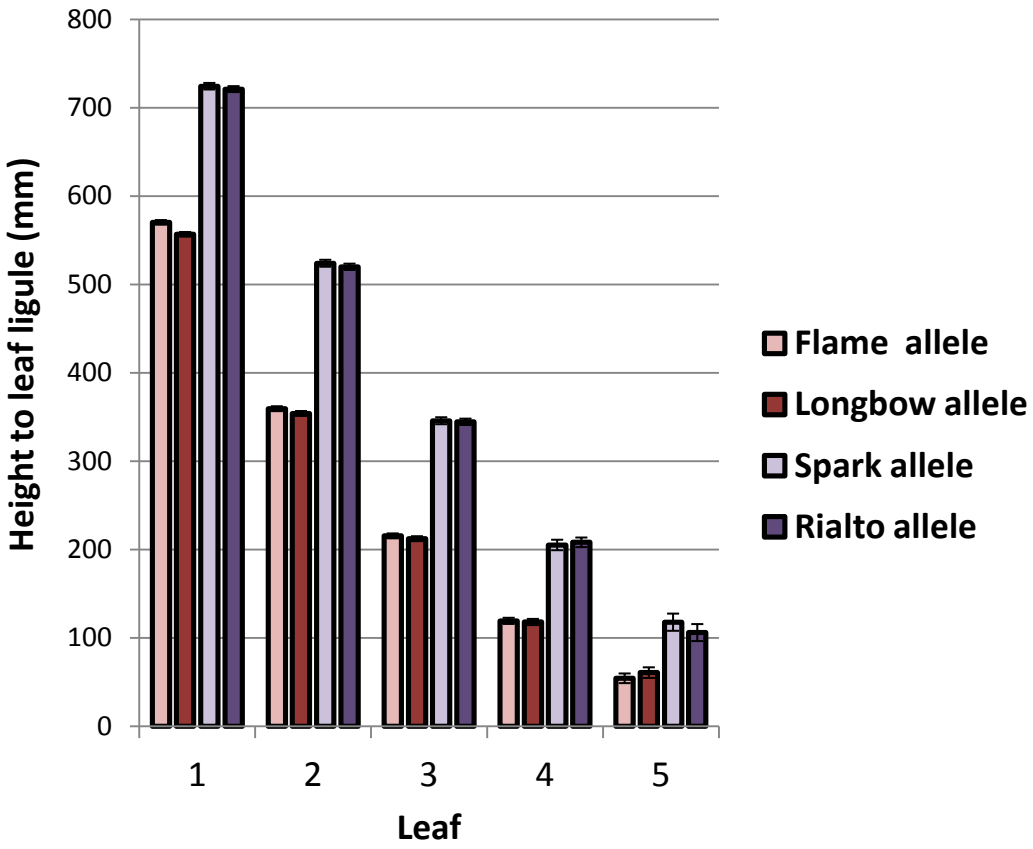

Figure 4.3.3: Predicted means for the height of the leaf ligules across all experiments where the trait was measured. The means were generated for the contrasting 6A alleles in the Flame x Longbow and Spark x Rialto NILs. Error bars are +/-1 standard error of the mean.

As with the overall plant height, there was a significant interaction between Family and Allele, for the heights of the individual leaf layers (Table 4.3.2). Whilst there wasn't an overall affect from the different alleles at the 6A locus, there was one in family 16 for Flame x Longbow (Figure 4.3.3). There was no significant interaction between the leaf and the allele indicating that the spacing between different leaf layers did not differ significantly between the NILs.

Variate: Ear length (mm)							
Model: (Year+Type+Location+Timepoint+Family)*Allele+Line+Unit							
Flame x Longbow NILs				**Spark x Rialto NILs**			
	d.f.	v.r	F pr.		d.f.	v.r.	F pr.
Year	3	256.39	<.001	Year	1	173.0851	<.001
Type	1	41.92	<.001	Type	1	1.37644	0.24
Location	2	163.84	<.001	Location	1	0.34411	0.56
Timepoint	6	12.16	<.001	Timepoint	4	3.284858	0.01
Family	1	0.93	0.34	Family	3	2.17423	0.10
Allele	1	3.98	0.05	Allele	1	8.473783	0.00
Year x Allele	3	2.27	0.08	Year x Allele	1	0.726781	0.40
Type x Allele	1	12.95	<.001	Type x Allele	1	2.322713	0.13
Location x Allele	2	0.97	0.38	Location x Allele	1	0.372325	0.54
Timepoint x Allele	5	0.83	0.53	Timepoint x Allele	4	0.239831	0.92
Family x Allele	1	0.05	0.83	Family x Allele	1	1.430755	0.23
Line	7	0.91	0.50	Line	3	5.300024	0.00
Unit	265			Unit	95		
Residual	535			Residual	428		

Table 4.3.3: Analysis of variance of the length of the ear in Flame x Longbow and Spark x Rialto NILs. Abbreviations as described in Table 4.3.1. The variance ratios were generated using the variance of the factor of unit instead of the residual.

The FLLO and SPRI backgrounds showed a significant relationship between the allele at the 6A locus and ear size (Table 4.3.3). However in FLLO this ear size difference varied in direction between experiments. In the field trials Longbow was associated with the increased ear length, whereas in the plastic glasshouse the Flame allele led to the greater size (Figure 4.3.4). Regardless of the type of experiment the Spark allele was associated with the large ears.

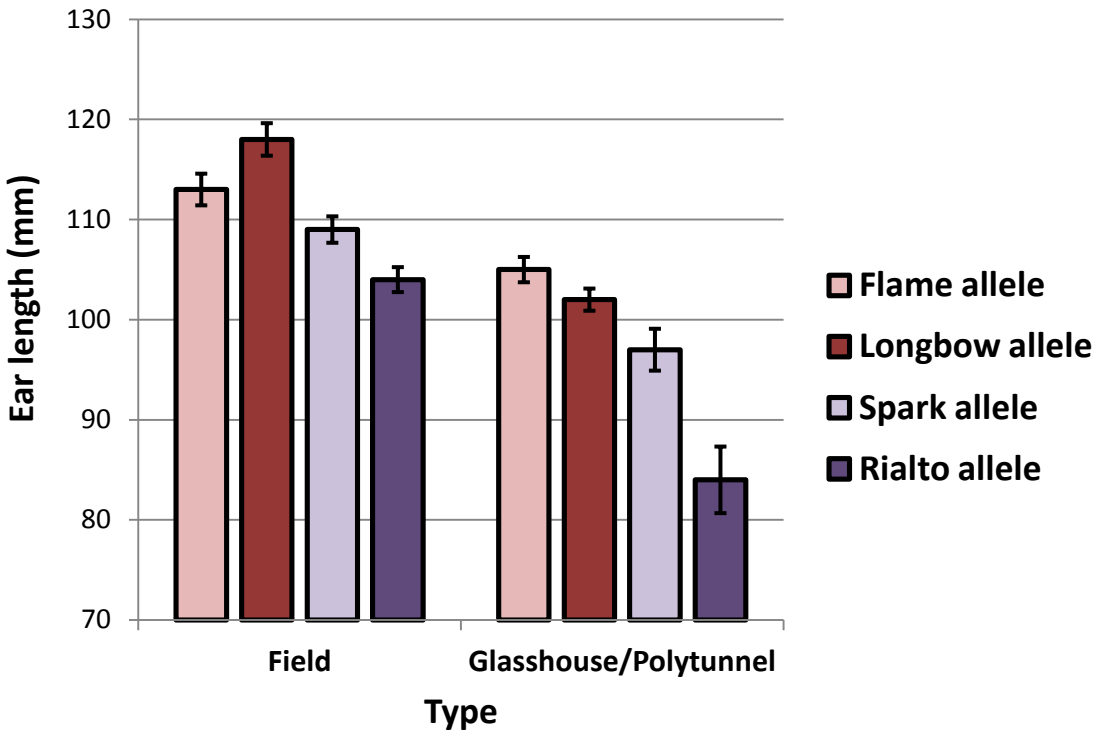

Figure 4.3.4: Predicted means for the length of the ears in both field and non-field experiments where the trait was measured. The means were generated for the contrasting 6A alleles in the Flame x Longbow and Spark x Rialto NILs. Error bars are +/- 1 standard error of the mean.

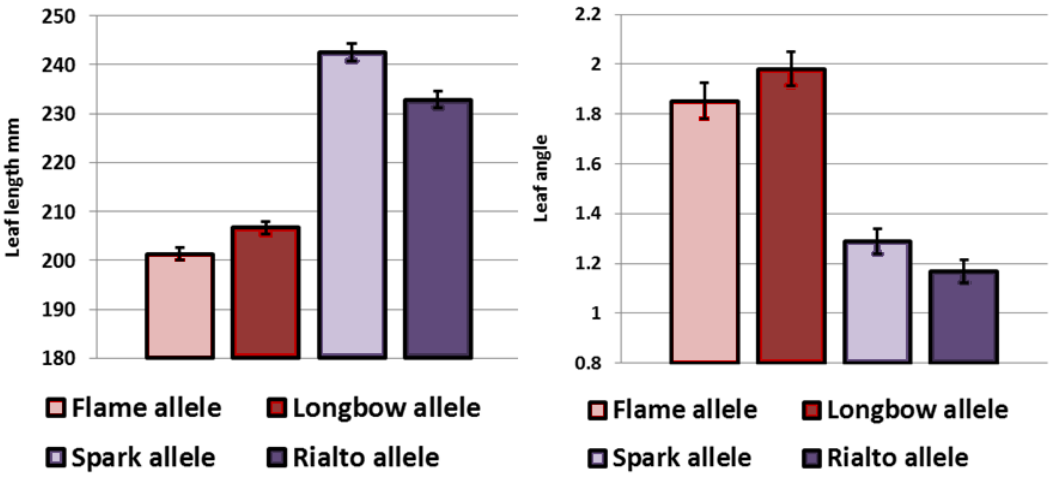

Figure 4.3.5a &b: Predicted means for the length (a) and angle (b) of the leaves across all experiments where those traits were measured. The means were generated for the contrasting 6A alleles in the Flame x Longbow and Spark x Rialto NILs. Error bars are +/-1 standard error of the mean.

The differences in leaf length caused by the alleles at the 6A locus showed opposing trends in the different genotypes (Figure 4.3.5a). The Longbow allele increased leaf size and the Rialto allele decreased it relative to the Flame and Spark alleles which share the maker Psp3071-167. The 2015 plastic glasshouse experiment showed results that differed from the previous years with the Flame allele being associated with the longer leaf length.

Variate: Leaf length							
Model: (Year+Type+Location+Timepoint+Leaf+Family)*Allele+Line+Unit							
Flame x Longbow NILs				**Spark x Rialto NILs**			
	d.f.	v.r	F pr.		d.f.	v.r.	F pr.
Year	3	485.03	<.001	Year	2	140.55	<.001
Type	1	313.97	<.001	Type	1	616.60	<.001
Location	3	161.07	<.001	Location	1	11.63	<.001
Timepoint	15	7.89	<.001	Timepoint	7	17.58	<.001
Leaf	4	80.50	<.001	Leaf	4	83.78	<.001
Family	1	3.99	0.05	Family	3	0.62	0.60
Allele	1	5.31	0.02	Allele	1	16.08	<.001
Year x Allele	3	1.57	0.20	Year x Allele	2	2.97	0.06
Type x Allele	1	3.34	0.07	Type x Allele	1	0.18	0.67
Location x Allele	3	0.64	0.59	Location x Allele	1	1.90	0.17
Timepoint x Allele	14	0.17	1.00	Timepoint x Allele	7	0.38	0.91
Leaf x Allele	4	0.39	0.81	Leaf x Allele	4	1.51	0.21
Family x Allele	1	1.75	0.19	Family x Allele	1	1.94	0.17
Line	7	0.70	0.67	Line	5	1.78	0.13
Unit	265			Unit	86		
Residual	2508			Residual	1079		

Table: 4.3.4: Analysis of variance of the leaf length in Flame x Longbow and Spark x Rialto NILs. Abbreviations as described in Table 4.3.1. The variance ratios were generated using the variance of the factor of unit instead of the residual.

Variate: Leaf Angle							
Model: (Year+Type+Location+Timepoint+Leaf+Family)*Allele+Line+Unit							
Flame x Longbow NILs				**Spark x Rialto NILs**			
	d.f.	v.r	F pr.		d.f.	v.r.	F pr.
Year	2	164.01	<.001	Year	1	0.98	0.33
Type	1	14.67	<.001	Type	1	0.37	0.54
Location	1	3.36	0.07	Location	1	1.23	0.27
Timepoint	1	0.56	0.46	Timepoint	1	0.50	0.49
Leaf	3	4.82	0.00	Leaf	3	8.75	<.001
Family	1	2.15	0.15	Family	3	0.88	0.46
Allele	1	1.22	0.27	Allele	1	2.85	0.10
Year x Allele	2	1.77	0.18	Year x Allele	1	0.04	0.84
Type x Allele	1	0.23	0.63	Type x Allele	1	1.11	0.30
Location x Allele	1	0.11	0.75	Location x Allele	1	0.22	0.64
Timepoint x Allele	1	0.31	0.58	Timepoint x Allele	1	0.40	0.53
Leaf x Allele	3	0.08	0.97	Leaf x Allele	3	2.21	0.10
Family x Allele	1	3.58	0.06	Family x Allele	1	1.52	0.22
Line	6	1.06	0.40	Line	3	1.44	0.25
Unit	77			Unit	37		
Residual	613			Residual	442		

Table: 4.3.5: Analysis of variance of the leaf angle in Flame x Longbow and Spark x Rialto NILs. Abbreviations as described in Table 4.3.1. The variance ratios were generated using the variance of the factor of unit instead of the residual.

The 6A locus didn't have a significant effect on the leaf angle (Table 4.3.5 & Figure 4.3.5b). Leaf angle was not a physiological trait that differed greatly between the experiments with the leaves having a generally erect stature in all time points and environments.

Variate: Leaf greenness (Spadmeter readings)							
Model: (Year+Type+Location+Timepoint+Leaf+Family)*Allele+Line+Unit							
Flame x Longbow NILs				**Spark x Rialto NILs**			
	d.f.	v.r	F pr.		d.f.	v.r.	F pr.
Year	3	279.22	<.001	Year	2	206.91	<.001
Type	1	465.50	<.001	Type	1	894.25	<.001
Location	2	23.87	<.001	Location	1	22.10	<.001
Timepoint	20	67.69	<.001	Timepoint	12	127.24	<.001
Leaf	4	569.14	<.001	Leaf	4	445.41	<.001
Family	1	2.39	0.12	Family	3	1.121	0.31
Allele	1	11.85	<.001	Allele	1	11.36	0.001
Year x Allele	3	3.13	0.03	Year x Allele	2	0.81	0.45
Type x Allele	1	2.62	0.11	Type x Allele	1	0.04	0.84
Location x Allele	2	0.82	0.44	Location x Allele	1	0.24	0.63
Timepoint x Allele	20	1.26	0.21	Timepoint x Allele	12	0.90	0.55
Leaf x Allele	4	0.15	0.96	Leaf x Allele	4	0.77	0.55
Family x Allele	1	0.57	0.45	Family x Allele	1	1.26	0.27
Line	6	2.63	0.02	Line	5	0.40	0.85
Unit	206			Unit	84		
Residual	4629			Residual	2048		

Table: 4.3.6: Analysis of variance of the leaf greenness (Spadmeter readings) in Flame x Longbow and Spark x Rialto NILs. Abbreviations as described in Table 4.3.1. The variance ratios were generated using the variance of the factor of unit instead of the residual.

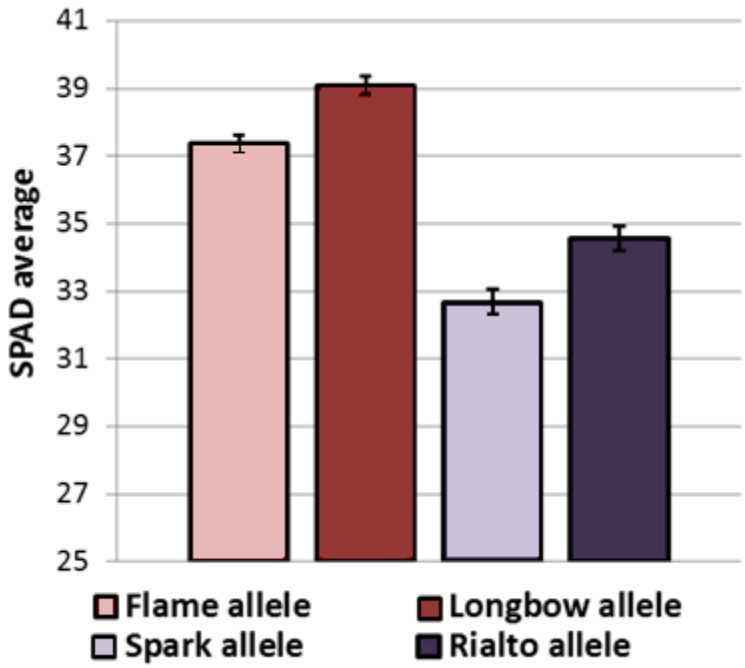

Figure 4.3.6: Predicted means for the average SPAD readings taken of the leaves across all experiments where leaf greenness was measured. The means were generated for the contrasting 6A alleles in the Flame x Longbow and Spark x Rialto NILs. Error bars are +/-1 standard error of the mean.

The 6A QTL has already been identified as having differences in canopy green leaf lifetime (Snape et al., 2007, Simmonds et al., 2014). This has been confirmed in the Spark x Rialto NILs, and is also shown to be significant in the Flame x Longbow material (Table 4.3.6 & Figure 4.3.6). As with leaf length, the physiological difference identified in previous years was reversed for FLLO in the 2015 plastic glasshouse experiment leading to Year x Allele being significant. Early physiology tests measuring % Senescence alongside SPAD readings showed the same effect, with successive time points measured showing an earlier loss of green leaf material in the Flame and Rialto lines. Simmonds et al (2014) only studied the SPAD readings of the flag leaf, but our data showed the same effect from the second to fifth leaf. This procedure was also studied in seedlings on the prophyll, 1st and 2nd leaf, with the difference in SPAD readings also being detectable in these leaves (Figure 4.3.7a)**.**

The use of the SPAD meter to detect levels of green pigment within the leaf did not differentiate between a difference in leaf lifetime and a shift in leaf emergence. Two

sets of experiments were used to identify which was occurring. Dark induced senescence tests showed no consistent differences in the senescence of the leaves, whereas repeated scoring of the leaf emergence of seedling leaves showed earlier development of leaves with the Flame allele at the QTL (Figure 4.3.7b). This indicated that the senescence of the leaves may happen earlier due to earlier emergence rather than a shorter leaf lifetime. SPAD readings were also performed on the Spark x Rialto recombinant lines (Figure 4.3.8).

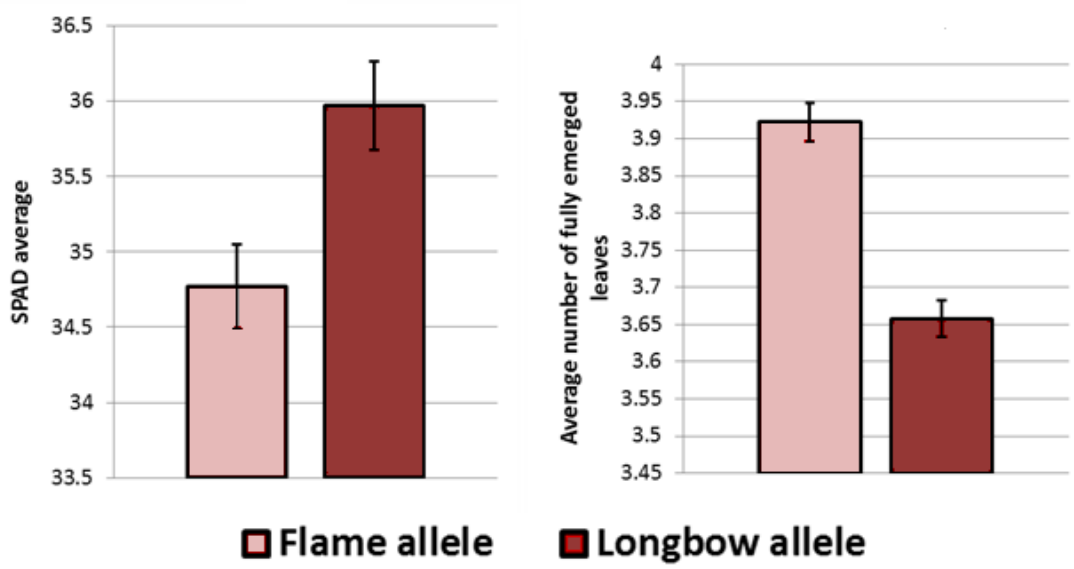

Figure 4.3.7 A&B: Predicted means taken from data collected from an experiment on seedling leaf development. A) Shows the average SPAD readings along the leaves, B) shows the average number of fully emerged leaves. The means were generated for the contrasting 6A alleles in the Flame x Longbow. Both graphs are averaged across multiple leaf layers and time points to show the overall effect of the alleles. This experiment consisted of nine seedlings grown from 40 of the homozygote genotypes identified in Table 8.1. Error bars are +/-1 standard error of the mean

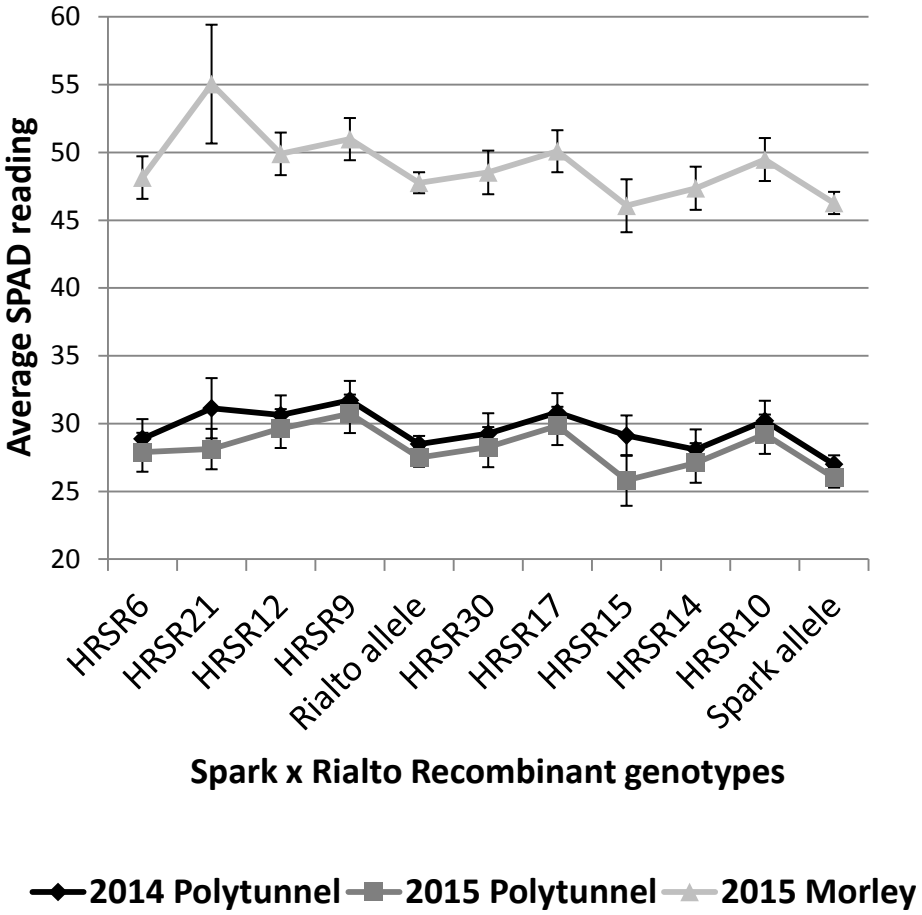

Figure 4.3.8: Predicted means for the average SPAD readings from the experiments including the SPRI recombinants. The means were generated for the different genotypes in the SPRI recombinants. The genotypes Spark allele and Rialto allele include the BC4 lines and the recombinants with the same allele configuration HRSR-9C and HRSR-10C. Error bars are +/-1 standard error of the mean.

Variate: Leaf area (Image J)							
Model: (Year+Type+Location+Leaf+Family)*Allele+Line+Unit							
Flame x Longbow NILs				**Spark x Rialto NILs**			
	d.f.	v.r	F pr.		d.f.	v.r.	F pr.
Year	1	61.33	<.001	Year	1	32.12	<.001
Type	1	673.41	<.001	Type	1	334.99	<.001
Location	1	0.40	0.53	Location	1	0.76	0.38
Leaf	3	33.06	<.001	Leaf	3	18.99	<.001
Family	1	41.19	<.001	Family	1	0.03	0.97
Allele	1	10.43	0.001	Allele	1	4.66	0.03
Year x Allele	1	8.07	0.004	Year x Allele	1	1.27	0.26
Type x Allele	1	0.77	0.38	Type x Allele	1	0.14	0.71
Location x Allele	1	7.63	0.01	Location x Allele	1	1.53	0.22
Leaf x Allele	3	0.52	0.67	Leaf x Allele	3	0.13	0.94
Family x Allele	1	2.56	0.11	Family x Allele	1	0.64	0.53
Line	4	2.00	0.10	Line	3	3.43	0.02
Unit	203			Unit	150		
Residual	818			Residual	863		

Table: 4.3.7: Analysis of variance of the leaf area (Image J) in Flame x Longbow and Spark x Rialto NILs. Abbreviations as described in Table 4.3.1. The variance ratios were generated using the variance of the factor of unit instead of the residual.

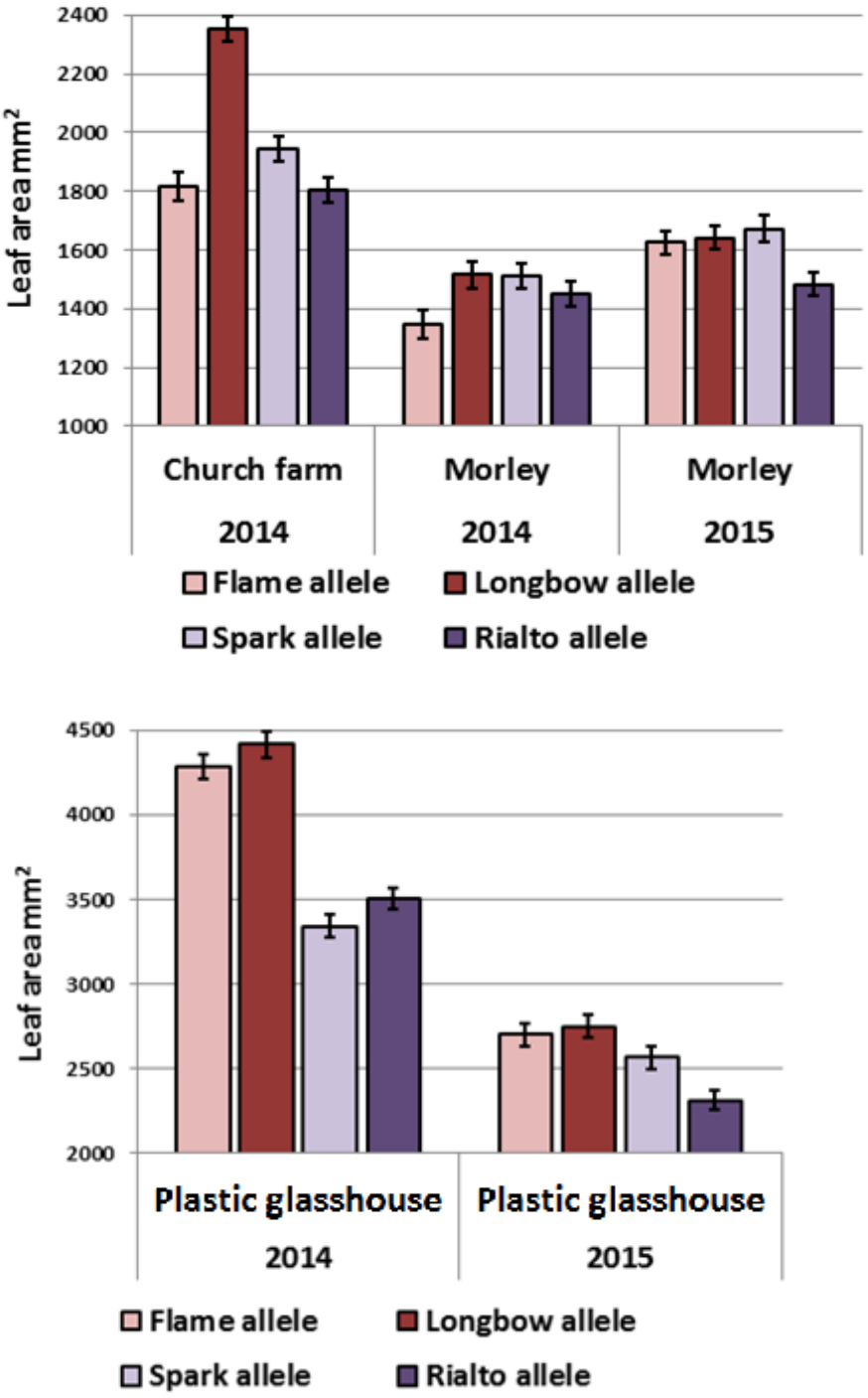

Figure 4.3.9 A and B: Predicted means for the leaf area of the leaves for each of the 2014 and 2015 experiments in the field (A) and plastic glasshouse (B). The means were generated for the contrasting 6A alleles in the Flame x Longbow and Spark x Rialto NILs. Error bars are +/-1 standard error of the mean.

Tests on leaf width in 2012 showed the leaves with the FLLO NILs with the Longbow allele at 6A were wider than those with the Flame allele. In 2013 the overall area was

measured for FLLO lines and showed that this width difference led to a significantly larger total area. In 2014 leaf area was still significantly larger for FLLO NILs with the Longbow allele in all three environments, although the significance was much lower in the plastic glasshouse than the field trials. The Spark x Rialto NILs didn't show any significant differences between the NILs in 2014 field trials but Rialto was associated with a small increase in leaf size in the plastic glasshouse (Figure 4.3.9). In 2015, this trend was reversed as the Spark allele led to larger leaves than the Rialto allele in both the field trial and plastic glasshouse. In 2015 there was no longer any significant difference attributable to 6A in the FLLO population in either the field trial or plastic glasshouse (Figure 4.3.9). These changing affects of the 6A locus on area are represented by the significance of Year x Allele and Location x Allele (Table 4.3.7). Leaf area readings were also taken for the SPRI recombinants (Figure 4.3.10). There was no apparent consistent shift in leaf area in relation to the position of the recombination break-point across the region of interest.

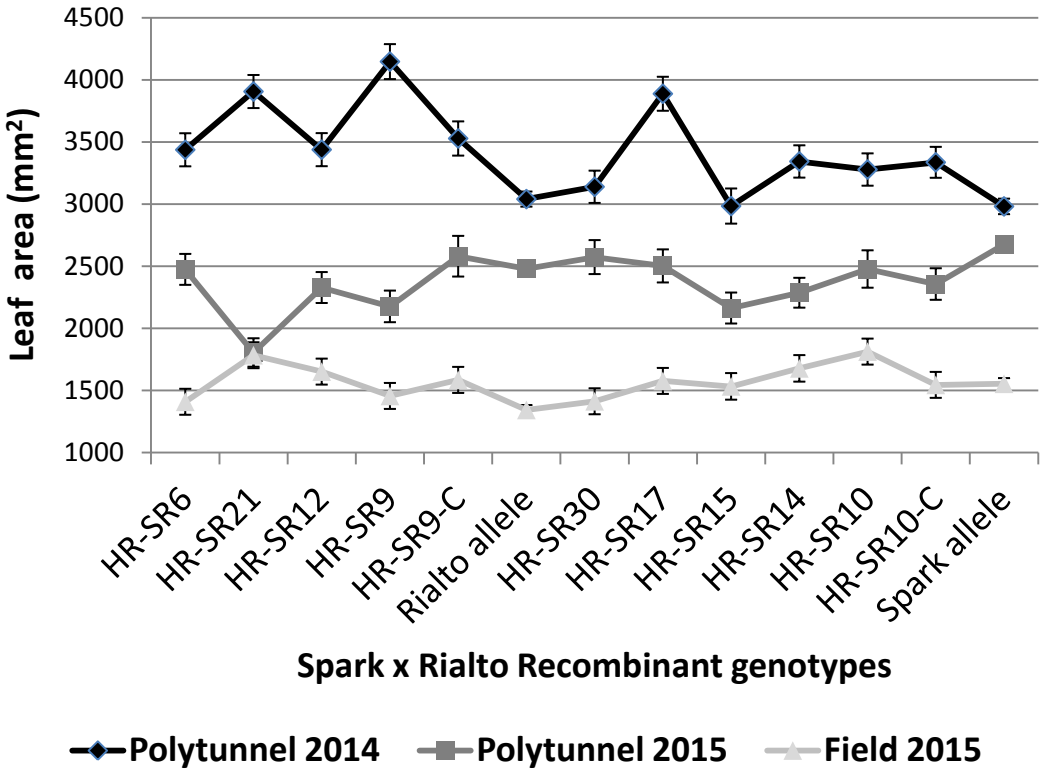

Figure 4.3.10: Predicted means for leaf area from the experiments including the SPRI recombinants. The means were generated for the different genotypes in the SPRI recombinants. The genotypes Spark allele and Rialto allele include the BC4 lines and

the recombinants with the same allele configuration HRSR-9C and HRSR-10C. Error bars are +/-1 standard error of the mean.

Minor traits	Experiment/Environment	FLLO	SPRI
Growth stage (Zadocks)	S63 2012 (GS1) S63 2012 (GS2)	-- --	* R>S * R>S
Ground coverage (% Green area)	Field 2014	--	--
Microclimate Temperature (^{0}C)	Field 2014 (Soil) Field 2014 (Canopy)	-- --	-- –
Seedling leaf emergence (No leaves emerged)	West 1 seedling tests 2013 Glasshouse seedling test 2013	* (F>L) **** (F>L)	* S>R
Seedling senescence (SPAD)	Glasshouse seedling test 2013 -Prophyll -First true leaf	 ****(L>F) ****(L>F)	Not tested
Dark induced Senescence (SPAD)	6 different seedling tests varying watering conditions and temperature.	--	--
Leaf fresh weight (g)	Field 2014 Plastic glasshouse 2014 Plastic glasshouse 2015	-- -- --	-- -- --
Leaf dry weight (g)	Field 2014 Plastic glasshouse 2014 Plastic glasshouse 2015	-- -- --	-- -- --
Heading	S54 2013 (Days to heading) Heading experiment 2014 (% emerged tracking main ear) Field 2014 (% emerged in a plot) Field 2015 (% emerged in a plot) RAGT 2014	****L>F *** L>F -- -- --	*** R>S *R>S -- -- --

Table 4.3.8: Summary of experiments testing other physiological and developmental traits. The statistical significance attributed to the factor "Allele" in the model. Each experiment/environment was analysed separately. The P value is represented as one of the following options (Blank: Not tested, --: 0.1<P, +: 0.05<P<0.1, *:0.01<P<0.05, **:0.005<P<0.01, ***:0.001<P< 0.005, ****: P<0.001). When there is a significant difference the allele that leads to the larger value is shown with a > symbol.

Table 4.3.8 shows the significance of traits tested that did were not appropriate to be included in the multiple experiment analysis. This may be due to them being tested in their own dedicated experiments (Dark induced senescence) or due to the same trait being measured using different methods. The extent of tillering was also measured in each experiment and differed between the different experiments and genotypes (Table 4.3.9).

	2012 54	***2013 53***	***2014 Church farm***	***2014 Morley farm***	***2014 Ireland***	***2014 Plastic glasshouse***	***2015 Morley farm***	***2015 Plastic glasshouse***
FLLO	*7.9*	*4.5*	*7.8*	*5.3*	*15-20**	*6.5*	*5.9*	*10.4*
SPRI		*5.6*	*10.5*	*6.9*	*15-20**	*9.1*	*6.4*	*12.9*

Table 4.3.9: Average tiller number for the FLLO and SPRI NILs in the different experiments. * is visual estimation rather than average. The Irish trial was sown at a far lower seed rate, due to diminishing seed stocks and a misunderstanding of size of plots to be sown.

Yield results

In 2014 the overall plot yield was not significantly affected by the presence or absence of the alternative alleles at the 6A locus (Table 4.3.10). However, the thousand grain weight (1000GW) was significantly greater in lines with the Longbow and Rialto allele (Table 4.3.10 & 4.3.11). This is in accordance with previous data collected on the SPRI NILs (Simmonds et al., 2014) but also identifies the same effect within the Flame x Longbow background (Figure 4.3.11). This difference in grain weight is due to an increase in grain width as opposed to grain length, agreeing with the established work on Spark x Rialto (Figure 4.3.12).

	Plot yield (Tonnes/Hectare)	1000GW (g)	Grain length (mm)	Grain Width (mm)
2014 Significance				
FLLO	-	****L>F	* L>F	**** L>F
SPRI	-	****R>S	-	****R>S
Hercules 2014 (S)	F:8.2 L:8.6 S:9.2 R:8.8	F:43.2 L:45.5 S:42.1 R:42.2	F:6.83 L:6.93 S:6.36 R:6.37	F:3.67 L:3.70 S:3.72 R:3.75
Track 2014 (S)	F:7.7 L:7.5 S:7.9 R:7.9	F:42.9 L:43.9 S:40.1 R:43.0	F:6.81 L:6.83 S:6.35 R:6.45	F:3.68 L:3.73 S:3.73 R:3.77
Ireland 2014 (S)	F:4.0 L:4.2 S:4.2 R:3.7			
Morley 2014 (S)	F:9.6 L:9.1 S:9.7 R:9.4	F:38.7 L:39.9 S:39.1 R:41.1	F:6.65 L:6.72 S:6.25 R:6.22	F:3.53 L:3.62 S:3.65 R:3.72
Morley 2014 (Y)	F:13.1 L:12.7 S:12.8 R:12.5	F:49.7 L:50.9 S:47.6 R:49.7	F:6.77 L:6.86 S:6.28 R:6.34	F:3.91 L:3.92 S:3.91 R:4.01
Track 2014 (Y)	F:9.3 L:10.1 S: 9.4 R:9.4	F:48 L:50.8 S:44 R:44.5	F:6.97 L: 7.01 S:6.40 R:6.36	F:3.84 L:3.89 S:3.84 R:3.86
RAGT 2014 (Y)	F:6.8 L:7.2 S:6.1 R:7.0	F:40.6 L:42.9 S:36.7 R:38.3	F:6.72 L:6.78 S:6.15 R:6.17	F:3.66 L:3.73 S:3.58 R:3.66
Limagrain 2014 (Y)	F:11.8 L:11.7 S:11.3 R:11.5	F:43.6 L:45.8 S:39.3 R:41.6	F:6.85 L:6.91 S:6.25 R: 6.25	F:3.79 L: 3.82 S:3.67 R: 3.75
2015 Significance				
FLLO	-	*L>F	-	-
SPRI	-	****R>S	-	**R>S
Morley 2015 (S)	F:10.5 L:9.98 S:10.9 R:11.0	F:39.9 L:40.7 S:10.9 R:11.0	F:7.06 L:6.96 S:10.9 R:11.0	F:3.56 L:3.53 S:3.38 R:3.62
Morely 2015 (Y)	F:12.9 L:12.6 S:12.3 R:12.6	F:49.8 L:52.0 S:12.3 R:12.6	F:7.24 L:7.25 S:12.3 R:12.6	F:3.92 L:3.97 S:3.81 R:3.97

Table 4.3.10: Summary of Yield data across field trial sites 2014/2015. Significance attributed to the factor of allele shown in accordance with preceding tables. (S) Indicates that the field was being used for disease assessment and thus received a low level of fungicide relative to the yield trials (Y). Predictive means were generated for the traits using the model **(Year+Field_Type+Field+Family)*Allele+Line** as shown for 1000GW in Table 4.3.11.

Variate: 1000 grain weight (g)							
Model: (Year+Field_Type+Field+Family)*Allele+Line							
Flame x Longbow NILs				**Spark x Rialto NILs**			
	d.f.	v.r	F pr.		d.f.	v.r.	F pr.
Year	1	0.68	0.412	Year	1	6.18	0.014
Field_type	1	325.17	<.001	Field_type	1	127.63	<.001
Field	6	45.48	<.001	Field	6	66.31	<.001
Family	1	244.93	<.001	Family	1	47.88	<.001
Allele	1	20.86	<.001	Allele	1	35.5	<.001
Year x Allele	1	0.51	0.477	Year x Allele	1	0.07	0.796
Field_type x Allele	1	0.52	0.47	Field_type x Allele	1	0.18	0.676
Field x Allele	6	1	0.425	Field x Allele	6	2.62	0.018
Family x Allele	1	5.69	0.018	Family x Allele	1	0.86	0.353
Line	4	3.12	0.016	Line	4	4.75	0.001
Residual	278			Residual	243		

Table 4.3.11: Analysis of variance of thousand grain weight in Flame x Longbow and Spark x Rialto NILs. Abbreviations as described in Table 4.3.1. Field type refers to whether the trial had fungicides applied to control STB.

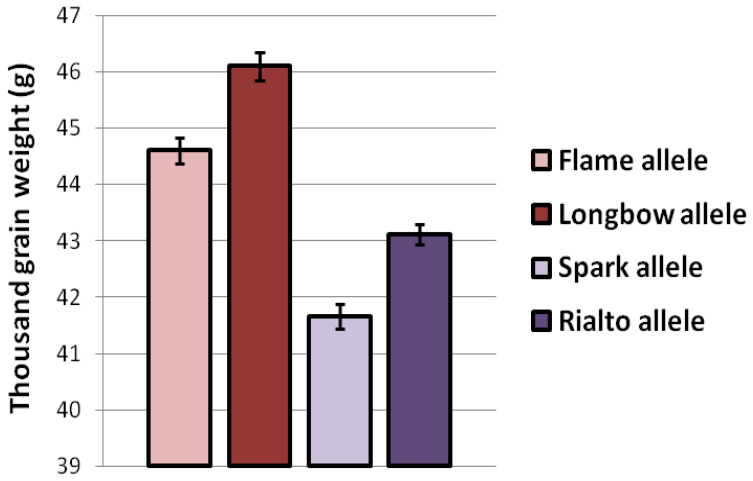

Figure 4.3.11: Predicted means for the 1000GW across the field trials. The means were generated for the contrasting 6A alleles in the Flame x Longbow and Spark x Rialto NILs. Error bars are +/-1 standard error of the mean.

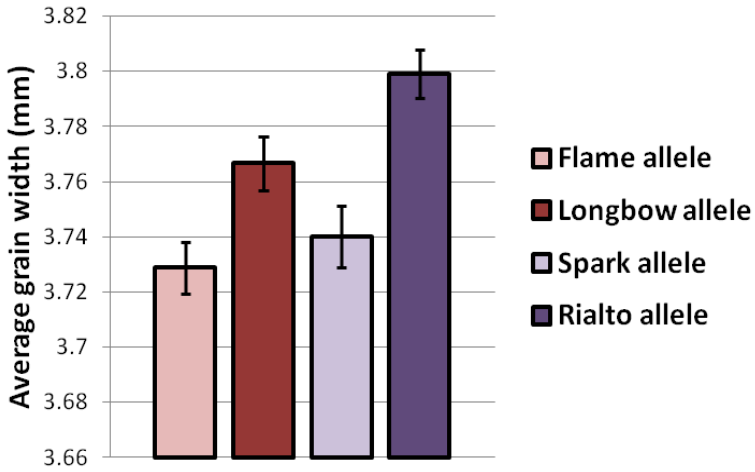

Figure 4.3.12: Predicted means for the grain width across the field trials. The means were generated for the contrasting 6A alleles in the Flame x Longbow and Spark x Rialto NILs. Error bars are +/-1 standard error of the mean.

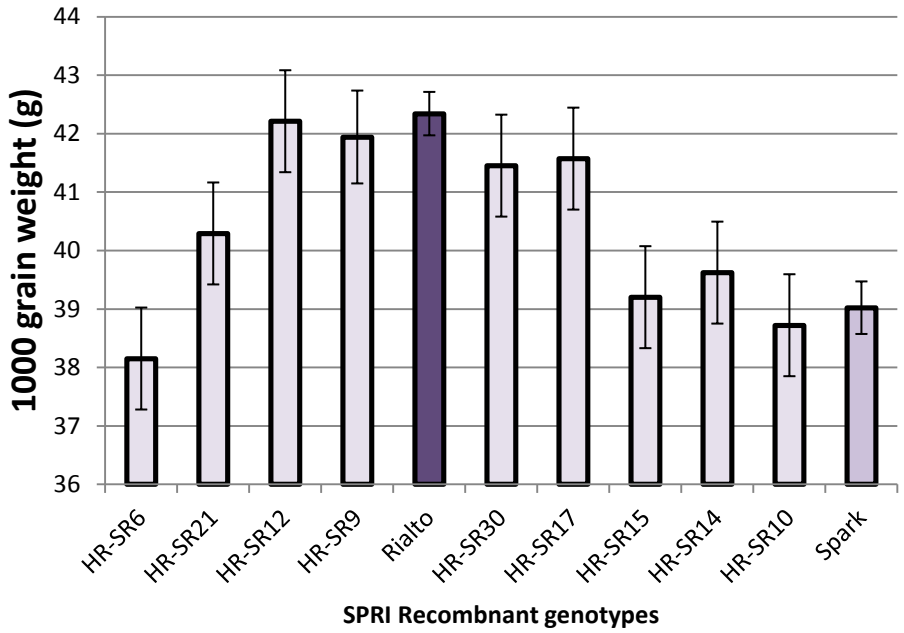

Figure 4.3.13: Predicted means for the 1000GW of the SPRI recombinants lines the 2015 field trials. The means were generated for the different genotypes in the Spark x Rialto NILs. Rialto refers to all the BC-4 lines with the Rialto introgression across all the tested markers across the 6A region and HR-SR9C. Similarly Spark is HR-SR10-C and the BC4 lines that resemble the Spark parent at each of the key markers. Error bars are +/-1 standard error of the mean.

The 2015 field trails included the Spark x Rialto recombinant lines. As with the other experiments, there were no significant differences in plot yield associated with the different genotypes, but there were differences for 1000GW. Four of the recombinant genotypes had significantly larger grains than the others (Figure 4.3.13). This indicates that the gene on chromosome 6A that effects yield is present in these recombinants but not in the neighbouring lines HR-SR21 and HRSR-15 (Table 2.3.2).

4.4 Discussion

Four main types of physiological trait were studied in this chapter, plant height, leaf physiology, leaf development & crop yield. All of these show a significant relationship with the alleles at the 6A locus in some way. Plant height showed significant differences associated with the 6A alleles in the FLLO family 16 but not in 24 or the SPRI background. This relationship was also found in the ligule heights which showed that the family 16 effect occurred in each leaf layer and was not a difference in final extension of the pedicule.

Leaf angle did not show a strong interaction with the 6A region. In the previous work indicating a link between leaf angle and STB (Lovell et al., 1997, Arraiano et al., 2009) the angles were more variable than those measured here (Figure 4.3.5b). Leaf area and its component traits of leaf length and leaf width showed significant differences in some experiments. From 2012-2014 the Flame x Longbow material had larger leaves when the Longbow allele was present. However this trait proved to be inconsistent and was absent in the 2015 experiments. The loss of leaf area significance in FLLO in the 2015 data can be explained with the switch in direction of the length effect in the plastic glasshouse and a loss of a length difference in the field. The increase in leaf area was also an increase in width as well as length, the 2012 width and length measurements showed a significant difference in width but not length. The Spark x Rialto material showed an increased leaf size for plants with the Spark allele in later experiments that was not significant in the preceding tests added further evidence that there was a large genotype by environment interaction for this trait. Tests on leaf fresh weight and dry weight showed no significant differences between the NILs implying that when the differences in leaf size occur, they are due to thinner leaves rather than an a increase in leaf biomass.

The leaf development was primarily assessed in terms of leaf senescence. SPAD readings and measurements of percentage senescence indicated that this trait was affected by the QTL from the earliest experiments. Seedlings studies on dark induced senescence and leaf emergence, support the concept of the difference in leaf

appearance during senescence is due to earlier leaf development rather than an alteration in leaf lifetime or the senescence process. This earlier development in the plants with the Flame and Spark alleles at the 6A locus fits with previous work showing BC4 NILs with the Spark allele reaching physiological maturity earlier than plants with the Rialto allele (Simmonds et al., 2014). However earlier development of the leaf layers in the Flame and Spark NILs was not identified by the assessment of ground cover in 2014. As with the leaf area data, the 2015 plastic glasshouse tests did not show the same relationship as the preceding years.

The differences in ear length did not align with the expected yield difference of increased yield for plants with the Longbow and Rialto allele. This supports the yield difference being solely due to changes in 1000GW and not an increase in grains per spike. Significant differences found in 1000GW and grain width between the NILS are in accordance with the previous work on the region (Simmonds et al., 2014), though they confirm that the QTL is resulting in the same phenotype in the Flame x Longbow backgrounds. It is more noteworthy that this change in 1000GW didn't lead to an increase in plot yield, implying that fewer grains per plot were produced. In Simmonds et al (2014), the BC4 NILs were associated with increased plot yield in four out five experiments, setting a precedent for the 1000 grain weight effect sometimes leading to no net yield increase. The recombinant lines show an increase in 1000GW for the recombinant lines on either side of the Rialto allele. The absence of the increase in either the SR-21 or SR-15 lines implies that the gene causing the yield increase is between psp3071 and BS00003581. There is no marker currently identifying a region that has Rialto alleles in SR12-SR17 but not in either of SR-21 or SR-15, however looking at the current marker data, the location of the gene can be predicted. This result indicates that the effect is not being caused by TaGW2 which has been previously linked to grain size increases (Zhang et al., 2013). The recombinant data for leaf area and leaf greenness didn't show a pattern that can be interpreted in the same way.

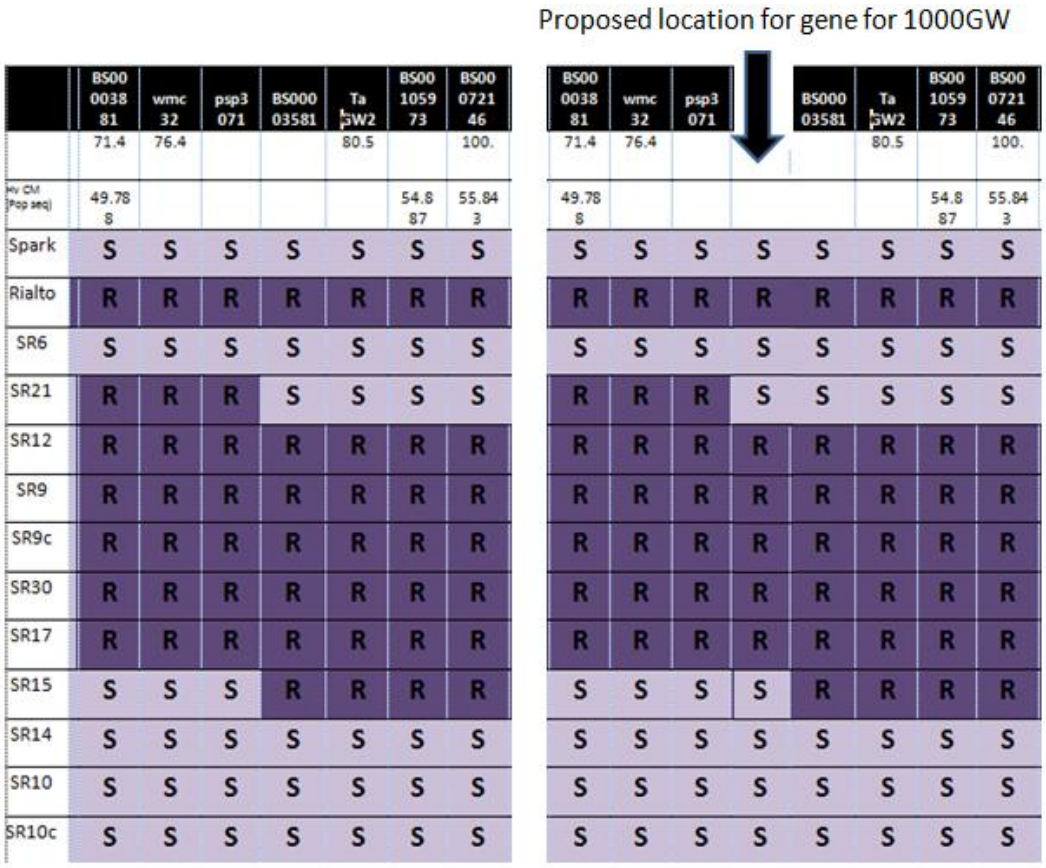

Figure 4.4.1: Predicted location for the yield gene based on the recombinant data.

There is not a marker currently identified where the five recombinant lines associated with increased 1000GW have material from Rialto, where the neighbouring recombinants do not. However the pattern of the recombinants indicates that the there may be genes that fulfil the criteria between PSP3071 and BS00003581.

Measuring wheat physiology and developmental traits for ideotyping purposes is often done in a far greater level of detail than this. These experiments were not designed to uncover detailed information about these traits themselves. Instead they were performed to identify if they were affected by the QTL and thus could be candidate traits for affecting the STB difference known for this region. Existing knowledge on this NIL only covered heading date, flag leaf lifetime and yield (Simmonds et al., 2014). These have now been confirmed in a separate population and many additional traits studied. The senescence differences have been shown operating in multiple leaf layers where it had previously only be studied on flag leaf. Seedling experiments have also indicated that this difference in SPAD readings may be due to shifted leaf emergence.

Previous literature on leaf area has not identified this region of chromosome 6A as affecting leaf size and despite the strong G by E interaction, there is still evidence that this region contributes to the determination of leaf area. The identification of a leaf area affect in the same region as a leaf yield QTL not only makes sense with the known importance of the flag leaf in grain filling but also has been shown to occur on chromosome 7A (Quarrie et al., 2006).

The main outcome of this chapter is how it relates to the others, as traits found in this region are not just having a source of variation identified, but are also raised as candidate disease escape traits. If any of them are the cause of the STB difference, this could lead to future work in understanding how they operate in more detail and on the economic tradeoffs involved.

Wheat plants are complex organisms with many physiological and developmental traits that can be measured. The selection of the ones chosen to study was based on the previous literature on disease escape and from visual observation of the NILs. However there was one trait observed to be different that there was not sufficient time and recourses to study. When the plants were grown in the field, differences in the colour of the plants could be seen that were not apparent in the glasshouse. This was only notable for the Flame x Longbow plants and was more so the ones from family 16. This may have been due to differences in the wax profile of the leaves, but the work required to test this was unfeasible when the difference was only detected in the field trials in late 2014. This trait also makes sense as a potential disease escape trait, as the waxiness of the leaf should affect the retention of rain droplets and spores on top of the leaves. As the detergent tween-20 is added to all inoculation experiments this difference wouldn't show up on the experiments in Chapter 3.

New technology looking at canopy cover would be ideal for studying this sort of system, such as the drones developed by companies like Precision Hawk. Our ground cover measurement was taken by photographing each plot at 1.5 m above the field trial plots then measuring the percentage of green pixels in the image. This method is old fashioned compared to those now being developed and may explain why there was no significant difference observed in terms of ground cover despite known

differences in leaf emergence. Whilst not being a separate trait, understanding how the crop canopy affects the microclimate would have been interesting to look at in more detail. A series of temperature readings were taken in the 2014 field trials, but modern sensor equipment that can be left in the field would have been able to build a greater understanding of how the differences in canopy traits may be affecting disease levels.

The traits studied showed variation in their effects between the different environments studied. Differences between field and glasshouse experiments may be explicable by density or age of plant. The plants in the glasshouses are grown with far greater spacing and are sown later in the year. Leaf size is already known to drastically differ between the two environments(Rebetzke et al., 2004). Plants grown in the plastic glasshouse in both years showed some stress responses from having low nutrient levels and being grown in a hot environment, these stress responses seemed to be greater in 2015.The field experiments were also subjected to different weather conditions with 2014 having greater rainfall than 2015.

The Spark x Rialto plants show fairly consistent trends in their physiology traits but the Flame x Longbow plants no longer showed a significant difference in leaf area in either of the 2015 experiments and showed a reversal of the SPAD difference in the 2015 plastic glasshouse. The change in the plastic glasshouse could be due to the aforementioned stress on the plastic glasshouse plants in 2015 or may be related to the different degree of tillering between the two years in the plastic glasshouse.

Summary

The 6A FLLO and SPRI NILs do not differ in their response to inoculation with STB (Chapter 3). Chapter 4 tested physiology and developmental traits in these lines and found that several showed significant variation affected by the QTL, but that there seems to be a strong interaction with the environment and background genotype. All traits tested were chosen as plausible causes of differences in disease escape. The differences in leaf length and area didn't show the same direction of trend with the Spark allele increasing leaf size in the SPRI lines, and Longbow increasing it in the FLLO material. The developmental data showed the same trend in all experiments except

the 2015 plastic glasshouse test and could lead plants with later emerging leaves getting more STB. The data on STB levels in each of these environments will be shown in Chapter 5 & 6, and can then be used to relate how the physiological and developmental traits in Chapter 4 do or do not relate to the STB scores found in the same location.

5 Effect of 6A locus on *Septoria tritici blotch* in field conditions

5.1 Introduction

In Chapter 3, the experiments showed that, within each family, near isogenic lines did not show significant differences in their response to inoculation with *Z.tritici.* The initial association genetics study identified this region as being very important for determining levels of STB in the field. Whilst physiological or developmental differences could lead to differences in disease escape (Lovell et al., 1997, Baccar et al., 2011), this hypothesis is predicated on the near isogenic lines differing in levels of STB and ideotype traits in the field. Despite the NILs being developed using psp3071 as the marker for selecting the lines, the differences in field STB levels associated with the marker may not be present in the NILs. The gene or genes that control the effect on STB are unknown and recombination may have occurred between them and psp3071. Alternatively the backgrounds used to develop the NILs may have interacted with the locus in a way that masked any effect. Therefore field trials of the NILs needed to be performed to test if the predicted disease differences occur within these lines.

Field trials of the lines can be used to test the effect of the 6A locus on flag leaf disease under natural infection. However to test the hypothesis it is also important to study the other levels of the canopy. Studying the disease in the lower leaves of the plant allows comparisons to be made between the seedling tests in the glasshouse and the natural infection of the seedlings. If the seedlings showed significant differences in the field, this indicates that resistance may be present in the NILs. However if the disease escape hypothesis is correct, it is predicted that there would be no significant differences between the 6A alleles in the lower leaves in the field. The escape hypothesis also predicts that differences between the NILs would emerge and increase towards the top of the crop canopy.

In this chapter, the 6A NILs response to natural infection with *Z.tritici* in the field is studied. The key questions addressed in this chapter are

- Do the NILs show the differences between the alleles as predicted by the association genetics study?
- Is the relationship between the allele and disease the same in the different backgrounds?
- Do STB levels in the lower canopy show similar results to the seedling tests?
- Does the progression of the STB though the canopies support the disease escape hypothesis.

5.2 Materials and methods

The purpose of performing field trials is to assess the traits of interest in conditions that closely resemble actual farming practice. Unlike laboratory experiments where the aim is to control as many variables as possible, it is good practice to have field trails in varied conditions. This enables a test of the robustness of the effects under different variables. Many traits in crops have strong interactions with the environment, which would not be identified without testing in multiple sites. This has to be balanced out by the practical concerns of running field trials, as they are both expensive and labour intensive. The trials performed in the project are listed in the table below.

Year	Trial Type	Location	Field ID	Management	Thesis Chapter
2013	Multiplication	Bawburgh, Norfolk	14AC	JIC	2
2014	Phenotyping/Yield	Bawburgh, Norfolk	Track Y	JIC	4
2014	Disease assessment	Bawburgh, Norfolk	Track S	JIC	4/5
2014	Disease assessment	Bawburgh, Norfolk	Hercules S	JIC	4/5
2014	Phenotyping/Yield	Morley, Norfolk	Morley Y	JIC	4
2014	Disease assessment	Morley, Norfolk	Morley S	JIC	4/5
2014	Yield	Cambridgeshire	RAGT Y	RAGT	4
2014	Yield	Cambridgeshire	LG Y	Limagrain	4
2014	Disease assessment	Dorset	Syngenta S	Syngenta	4/5
2014	Disease assessment	Carlow. Ireland	Teagasc S	Teagasc	5
2015	Phenotyping/Yield	Morley, Norfolk	Morley Y	JIC	4
2015	Disease assessment	Morley, Norfolk	Morley S	JIC	4/5

Table 5.2.1: List of field trials used in the project. S in the field ID means that the trial was untreated with fungicides that affect STB, to favour infection. Y in the field ID means that the trial had a regiment of treatments applied by the team managing the trial to minimise yield loss from disease.

Six of the field trials were used to assess the levels of infection by *Z. tritici* in the 6A NILs. Data from these experiments form the results for this chapter. Selection of the trial sites was influenced by numerous factors. In 2013 there wasn't sufficient material available for disease assessment, so the lines were bulked up in the multiplication trial. In 2014, the intention was to gain as much data as possible, as seed was readily

available. Three different disease trials were performed in Norfolk. This was because the John Innes Centre already has access to fields in these locations to perform trials. In addition it was important to have several trials close by to allow for regular access for observation and scoring. This enabled more detailed and regular measurements to be made, than could be done with trials further apart. However it was also important to have trials in other locations as STB levels were likely to be lower than in other parts of the country as Norfolk has typically lower rainfall levels than the rest of the UK. Additional Septoria trials were hosted by Syngenta and Teagasc in Dorset and Carlow respectively in 2014. In 2015 an additional set of experiments was performed at Morley, Norfolk as additional repeat of the experiment.

John Innes Centre field trials

The field trials, run in conjunction with the John Innes Centre, were all performed with the same specifications. The plots were sown at a rate of 260 seeds/m^2 in 6m x 1.5m plots. These plots were subsequently cut back to 4m x1.5 m. The resultant 6m^2 plots in the STB trials were then managed to favour STB development. This meant not using triazole, SDHI or chlorothalonil fungicides as they retain some effectiveness against STB. The trials were regularly monitored and checked with the assistance of the field trials team. Each of the three 2014 trials were designed to include two blocks, with each containing the 15 major NILs (Table 2.2.2). The plot randomisations for these trials are shown in Chapter 2 (Figure 2.3.6-10). The 2014 trials were sown in 2013 with the trials at Morley being sown first on the 23rd of September with Track and Hercules being sown on 3rd and 4th October respectively. On each of the JIC disease assessment field trials, notes were made on the infection levels of other diseases, and height was scored due to the inclusion of the height variability lines within the same trial. The 2015 trials at Morley were run in the same manner as the 2014 work and were sown on 26th September 2014**.** The only major change was the removal of the height variability lines from the trial and the inclusion of SPRI recombinant lines (Figure 2.3.11-12).

Assessment of STB in the field is frequently performed by taking visual assessments of the entire crop canopy at once. To gain a greater amount of detail on the disease levels

within the different plots, multiple leaves were assessed individually. As in the glasshouse tests, Septoria leaf blotch was scored on individual leaves as the percentage of the leaf bearing lesions containing pycnidia (Chapter 2.6)**.** To study disease levels at different heights within the crop, the leaves were sampled at random within different height ranges. This was done by height rather than leaf number as judging leaf number correctly before flag leaf emergence is difficult, and it should partially account for the height differences between the different genotypes. The five height ranges were S1 (0mm – 100mm), S2 (100mm-250mm), S3 (250mm-350mm), S4 (350mm-500mm) and S5 (500mm+). Plants for scoring were selected from the plot at random, and then the main tiller had a leaf scored in each height range. The position was determined based on the height where the ligule joins the stem. If there were multiple leaves within each range, the highest in the region was selected. For the majority of the readings there was only one leaf within the regions, but this method means that S5was always the flag leaf after it had emerged, even if the second leaf was within the same height range. For each of these height ranges, ten readings were taken from each plot at random. In 2014 scoring was performed 7-8 times throughout the year, with one reading being taken from each trial in December and March, followed by two readings in May, June and July. Due to adverse weather conditions readings could not be gathered for the Hercules trial in December. In 2015, scoring was performed four times in late May, early June, late June and early July, as these time points corresponded with when the disease was developing most. For these time points in 2015 an additional score was taken measuring the height of the highest lesion of septoria on each plant sampled. These time points were scored with the help of a casual worker, Benjamin Gibson. The sampling for each trial is summarised in Table 5.2.2.

Trial	Score types	Readings per score per plot	No of Plots	Time points	Readings per Allele per score
Hercules S	S1-S5	10	72	6	360-480
Track S	S1-S5	10	72	7	420-560
Morley S 2014	S1-S5	10	72	7	420-560
Morley S 2015	S1-S5 Max Height	10	71	4	360-480
Teagasc S	Flag & 2nd	10	30	2	120-160
Syngenta S	Whole plot	1	15	2	6-8

Table 5.2.2: Summary of sampling in the STB field trials. The lower number in readings per allele per score is the number of readings with the Spark allele. The higher number is the number of readings for Flame, Longbow and Rialto. There are less Spark readings due to the missing line from bunt contamination.

Ireland Field trial (Teagasc S)

Seed of each of the 15 main NILs were sent to Teagasc for trialling. An important difference between this trial and the JIC trials was the size of the plots, which were 12 x 3 metres, as opposed to the 6 m^2 used at JIC and the sowing rate was 280 seeds/m^2. This was lower than usual for seeds sown late in November, but the large plot size and limited seed stocks meant that a higher rate couldn't be used. The trial management was performed by Teagasc. Scoring of STB was performed by Cliona Connolly (Teagasc) and Margaret Corbitt (JIC) on 18st and 23rd June 2014. Scores were performed by sampling 10-20 leaves per plot, on the top three leaf layers. Then each leaf was scored according to the standard STB scoring protocol (Chapter 2.6). Whilst less detailed than the S1-5 method, it still separates the canopy into multiple scores

Syngenta field trial

The lines that were sent to Syngenta were incorporated into an STB trail they were performing on other lines. Their experimental protocol was to grow the crop in plots of 1 square metre and there was only space for 1 replicate. Our NILs were scored for STB twice. STB was scored at the level of the whole plot, with a percentage infection being recorded by someone trained to recognise the symptoms. Whilst this method

differs from the multiple leaf scores method used in our other trials, it is standard industry practice to assess levels of STB with this method. In addition to STB, other diseases were scored on the lines, as a matter of practice when performing large scale assessments. Unfortunately this trail became so heavily infected with Yellow Rust (*Puccinia striiformis*) that it was not possible to obtain STB data of acceptable quality from it.

5.3 Results

JIC Trials

The disease scores at each height level in the JIC run trials were combined together to perform the analysis of the data. Plants of both Flame x Longbow families showed significant differences in the levels of STB in the uppermost canopy (S5) of the crop, associated with the alleles at the 6A locus. Disease levels at lower heights were not significantly affected by the different alleles (Table 5.3.1 & Figure 5.3.1). This effect was found in every field environment tested in 2014 and 2015 (Figure 5.3.2). Family 16 had higher levels of STB than those from 24, but this did not interact with the different alleles, indicating that the difference was due to variation in the genetic backgrounds of the two families.

	FLLO S1			FLLO S2			FLLO S3			FLLO S4			FLLO S5		
Factor	d.f	m.s	f.pr	d.f	m.s	f.pr	d.f	m.s	f.pr	d.f	m.s	f.pr	d.f	m.s	f.pr
Year	1	349.03	<.001	1	204.61	<.001	1	34.65	<.001	1	63.36	<.001	1	52.58	<.001
Site	1	34.87	<.001	1	59.76	<.001	1	27.64	<.001	1	59.87	<.001	1	28.33	<.001
Field	1	170.97	<.001	1	217.61	<.001	1	138.91	<.001	1	93.55	<.001	1	35.95	<.001
Timepoint	7	171.29	<.001	7	1406.81	<.001	7	1281.93	<.001	7	792.00	<.001	7	771.36	<.001
Family	1	1.18	0.28	1	13.90	<.001	1	11.94	<.001	1	36.26	<.001	1	47.36	<.001
Allele	1	0.15	0.70	1	0.85	0.36	1	0.01	0.92	1	1.26	0.27	1	3.65	0.05
Year x Allele	1	0.92	0.34	1	1.02	0.32	1	1.95	0.17	1	1.15	0.29	1	1.12	0.29
Site x Allele	1	0.90	0.34	1	0.24	0.63	1	0.00	1.00	1	0.03	0.86	1	0.10	0.75
Field x Allele	1	0.04	0.85	1	3.00	0.09	1	0.21	0.65	1	0.09	0.76	1	0.17	0.68
Timepoint x Allele	7	0.89	0.52	7	0.45	0.87	7	0.15	0.99	7	0.33	0.94	7	1.47	0.19
Family x Allele	1	0.00	0.95	1	2.51	0.12	1	0.08	0.78	1	0.27	0.60	1	0.09	0.77
Line	6	0.62	0.71	6	0.50	0.80	6	0.59	0.74	6	0.49	0.82	6	0.50	0.81
Field.Plot	81			81			81			81			81		
	SPRI S1			SPRI S2			SPRI S3			SPRI S4			SPRI S5		
Factor	d.f	m.s	f.pr	d.f	m.s	f.pr	d.f	m.s	f.pr	d.f	m.s	f.pr	d.f	m.s	f.pr
Year	1	399.84	<.001	1	164.71	<.001	1	105.51	<.001	1	236.72	<.001	1	508.72	<.001
Site	1	26.33	<.001	1	27.74	<.001	1	17.26	<.001	1	63.08	<.001	1	25.48	<.001
Field	1	149.17	<.001	1	154.82	<.001	1	67.67	<.001	1	49.84	<.001	1	61.71	<.001
Timepoint	7	153.96	<.001	7	1201.65	<.001	7	691.81	<.001	7	817.73	<.001	7	1491.54	<.001
Family	3	0.10	0.96	3	0.92	0.43	3	0.71	0.55	3	1.75	0.16	3	2.79	0.05
Allele	1	0.33	0.57	1	0.00	0.95	1	0.76	0.39	1	0.01	0.93	1	0.02	0.88
Year x Allele	1	0.09	0.76	1	0.14	0.71	1	3.42	0.07	1	0.96	0.33	1	0.04	0.84
Site x Allele	1	0.04	0.85	1	0.13	0.72	1	0.01	0.94	1	0.25	0.62	1	0.00	0.97
Field x Allele	1	0.26	0.61	1	0.76	0.39	1	0.02	0.89	1	0.06	0.81	1	0.24	0.63
Timepoint x Allele	7	0.16	0.99	7	0.55	0.80	7	0.75	0.63	7	1.25	0.29	7	0.92	0.49
Family x Allele	1	0.07	0.79	1	0.00	0.95	1	0.10	0.75	1	0.67	0.41	1	0.64	0.43
Line	3	0.08	0.97	3	0.09	0.97	3	0.90	0.44	3	1.20	0.31	3	0.66	0.58
Field.Plot	77			77			77			77			77		

Table 5.3.1: Analysis of variance table for the scores of STB in JIC run field trials. The data are separated into the two populations Flame x Longbow (FLLO) and Spark x Rialto (SPRI), and into five different height ranges. The height ranges for STB scoring were S1 (0mm – 100mm), S2 (100mm-250mm), S3 (250mm-350mm), S4 (350mm-500mm) and S5 (500mm+).The analysis was performed on logit adjusted STB scores with the model (***Year+Site+Field+Timepoint+Family)*Allele+Line+Field/Plot***.** Logit transformation performed as described in Table 3.3.2.The between-plot variance was used for the calculation of the F probabilities as it is the relevant unit of experimentation for field trial data.

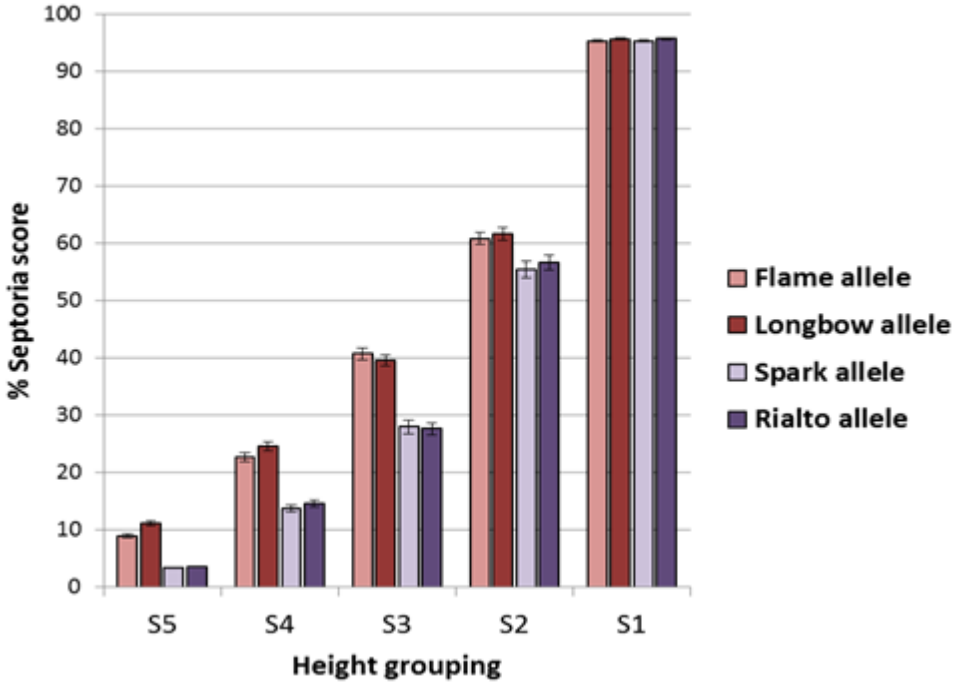

Figure 5.3.1: Average overall scores of STB in JIC field trials. The data is separated into which parent contributed the allele at the 6A locus in the two populations Flame x Longbow (FLLO) and Spark x Rialto (SPRI). The height ranges for STB scoring were S1 (0mm – 100mm), S2 (100mm-250mm), S3 (250mm-350mm), S4 (350mm-500mm) and S5 (500mm+). Values shown are predicted means from the model in Table 5.3.1. Error bars are +/-1 standard error of the mean.

The Spark x Rialto population did not show significant differences in levels in STB in any of the different height ranges (Table 5.3.1 & Figure 5.3.1). This was even the case in the highest part of the canopy (S5), although a slight trend in the expected direction can be seen in the data from Track field (Figure 5.3.2). Recombinant lines developed by James Simmonds and the Uauy lab for the SPRI population were included in the 2015 trial at Morley. Although the lines with the Rialto and Spark genotypes across the whole region not differ significantly, there was a difference between the recombinants. The HR-SR6 and HR-SR21 genotypes have a Spark segment at psp3071, but a Rialto segment nearby on 6A (Table 2.3.2). They also had increased levels of STB

compared to the other recombinant lines (Figure 5.3.3). This difference was also identifiable at S4 but is absent in the lower leaf levels.

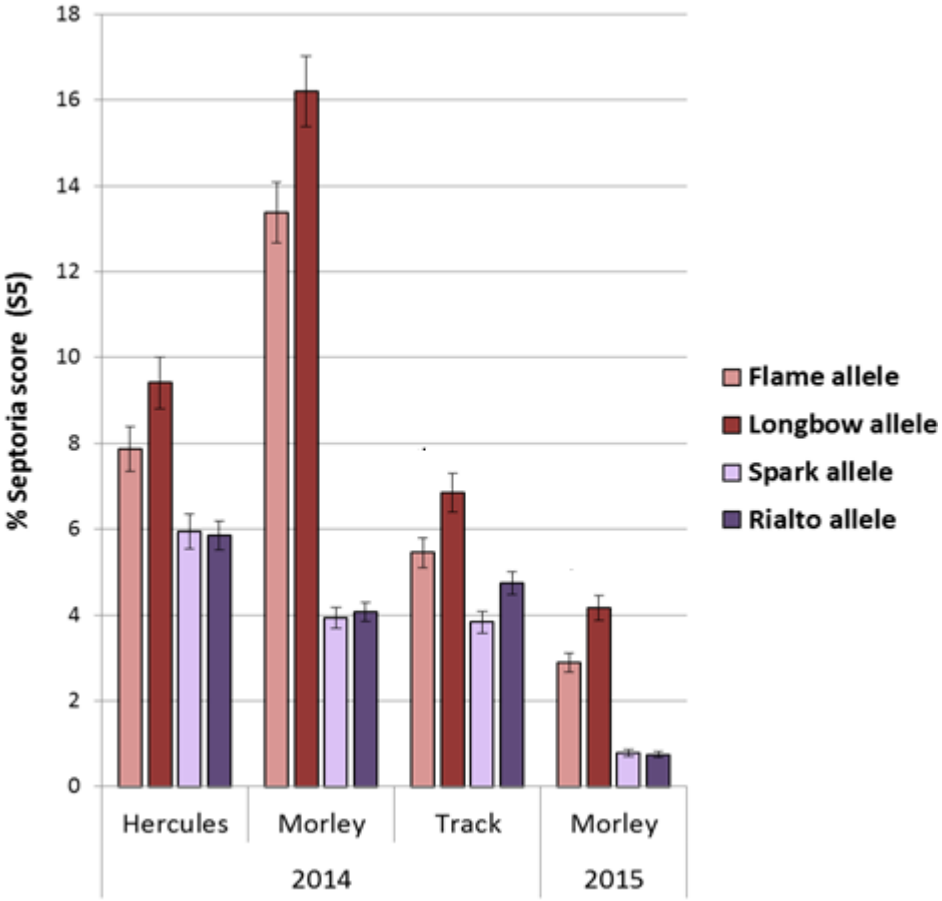

Figure 5.3.2: Average scores of STB in the upper canopy of the different JIC field trials. The data is separated into the different trials and by which parent contributed the allele at the 6A locus in the two populations Flame x Longbow (FLLO) and Spark x Rialto (SPRI). The S5 height range scores the highest leaf above 500mm, for the majority of time points this will be the flag leaf. Values shown are predicted means from the model in Table 5.3.1. Error bars are +/-1 standard error of the mean.

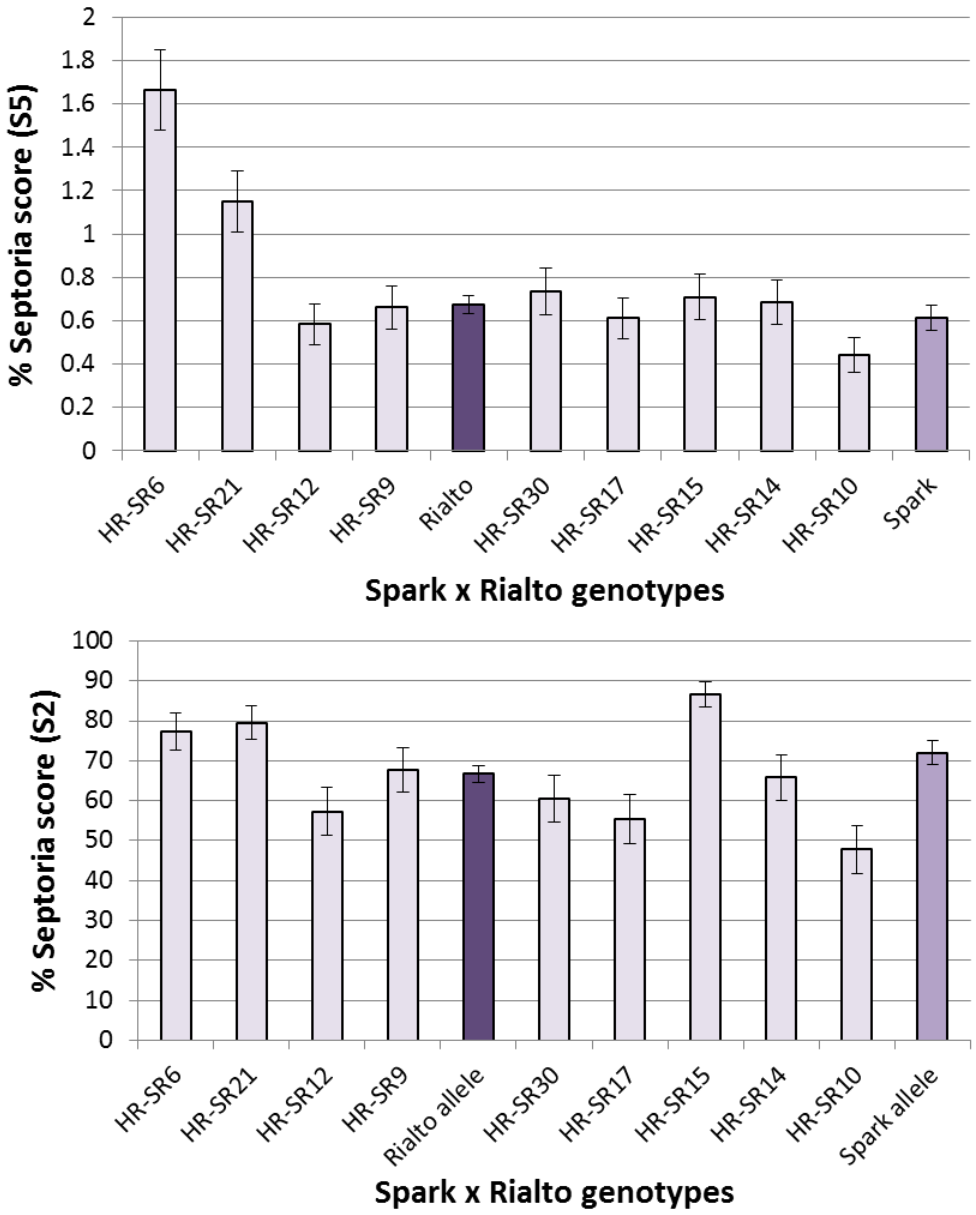

Figure 5.3.3: Average scores of STB for SPRI recombinant lines in the S2 and S5 height categories. The data is from the 2015 STB trial at Morley, Norfolk. The genotypes listed refer to different combinations of Spark x Rialto (SPRI) material on the 6A chromosome. The Rialto allele and Spark allele data points include multiple lines that were uniform in the parent which contributed the material in the region. The S5 height range scores the highest leaf above 500mm, for the majority of time points this will be the flag leaf. The S2 category includes leaves between 100mm and 250 mm from the ground in the base of the canopy. Values shown are predicted means from the model **(Timepoint+Family)*allele+Line+Plot** on logit STB scores. Error bars are +/-1 standard error of the mean.

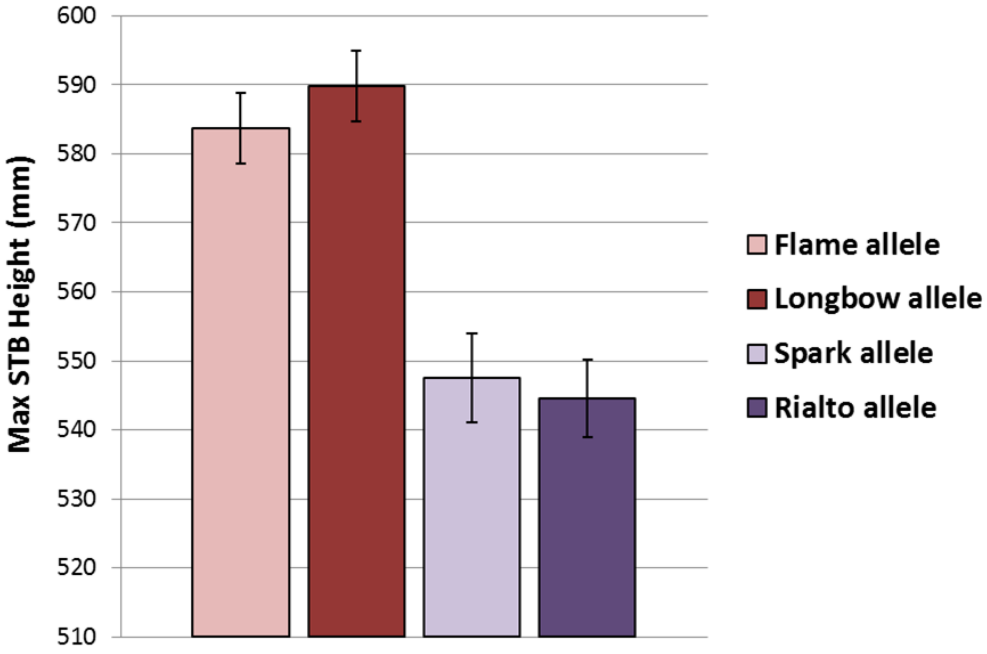

Figure 5.3.4: Max STB Height in 2015 field trail. The data is from the 2015 STB trial at Morley, Norfolk. In this trial when the field was scored, the height of the highest STB lesion on the plant is measured as max STB height. The data is separated by which parent contributed the allele at the 6A locus in the two populations Flame x Longbow (FLLO) and Spark x Rialto (SPRI). Values shown are predicted means from the model **(Timepoint+Family)*allele+Line+Plot**. Error bars are +/-1 standard error of the mean.

In the 2015 STB trial, the height of the highest STB lesion ('max STB height') was recorded for each plant sampled. Timepoint was a significant factor in both backgrounds with each successive measurement showing the STB at a higher point in the crop. The max STB height scores did not show significant differences caused by the different alleles (Figure 5.3.4). However the two FLLO backgrounds showed significant differences with family 16 having STB higher in the crop than family 24 at all timepoints.

Teagasc trial

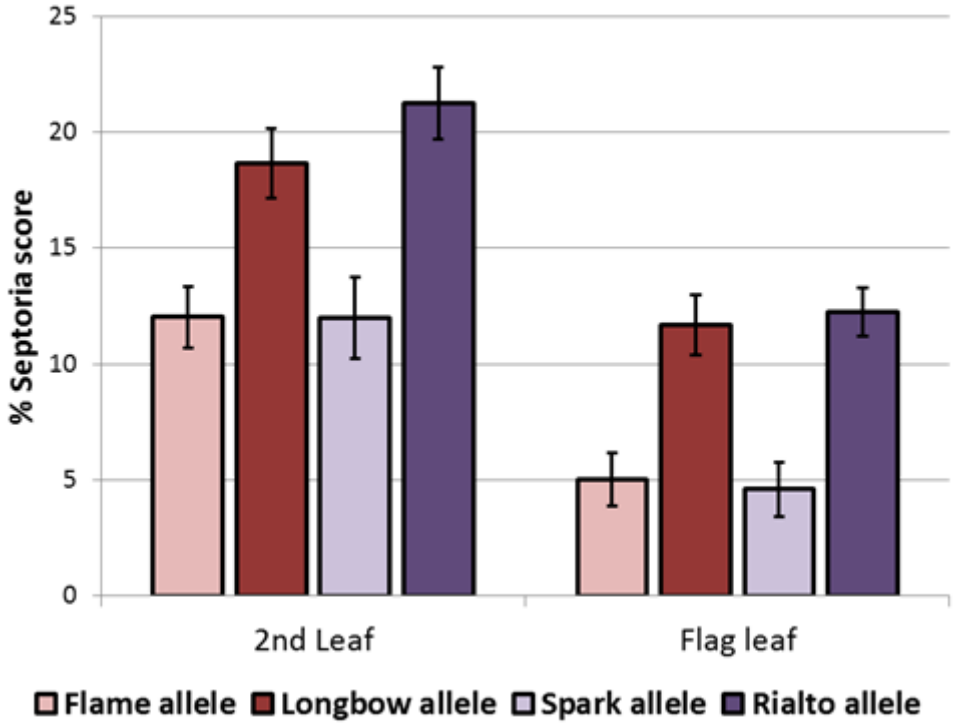

Figure 5.3.5: STB levels from Teagasc trial in 2014. The data is separated by the measured leaf layers and by which parent contributed the allele at the 6A locus in the two populations, Flame x Longbow (FLLO) and Spark x Rialto (SPRI). Values shown are the predicted means from the model **(Timepoint+Family)*allele+Line+Plot**. Error bars are +/-1 standard error of the mean.

In both populations, the Teagasc trial showed significant differences in levels of STB, (Figure 5.3.5). NILs with the Longbow or Rialto allele at 6A had greater levels of STB in the FLLO and SPRI NILs respectively. This was a much smaller experiment than the work done at JIC, and was sown at a lower density in an area with higher disease pressure. The two backgrounds also gave similar levels of overall disease in this trial.

5.4 Discussion

The lack of a significant effect of the 6A locus on STB in the low height ranges in either the FLLO or SPRI background is consistent with the lack of significant differences in resistance in inoculated seedlings and adult plants (Chapter 3). If the 6A locus affects disease escape, it would be predicted that the there would be differences in STB levels in the canopy but not necessarily on the lower leaves. This was the case for the Flame x Longbow NILs, which showed a significant difference in STB in the topmost level of the crop, S5, in all environments and years. This confirms that there is a difference in field STB controlled by the 6A region as predicted by the initial association genetics study. The presence of a significant difference in STB on the flag leaf but not lower leaves could be due to disease escape, or due to resistance that develops only on the upper leaves. As direct inoculation of the flag leaf in experiments AP1 & AP2 did not lead to significant differences in STB (Figure 3.3.8, & 3.3.10), it is concluded that the 6A allele of Flame contributes to increased disease escape rather than greater adult plant resistance.

Surprisingly, the 6A effect was not observed in SPRI in the JIC trials but the Rialto allele in the 6A region was associated with higher STB scores in the Irish trial. Possible explanations for these findings include the following.

1) There is no effect on STB caused by the 6A region, but there is another region in the FLLO NILs that causes an escape difference

The fact that the NILs from both FLLO families 16 and 24 show the STB differences makes this explanation implausible. The two families were bred from independent F2 plants, and allelic differences in a gene other than that linked to Psp3071 would have had to have been maintained for four generations in the correct phase. The probability of this happening in both families is low. The significantly greater levels of STB associated with Rialto in Ireland, also indicates that this explanation is incorrect.

2) Differences in size between the NILs mean that the FLLO NILs differ in alleles affecting STB but the SPRI ones do not.

The presence of a significant STB difference associated with the 6A locus in the Teagasc trial does not support this explanation. In addition the tested markers indicated that the introgressed region in the FLLO NILs is smaller than that in the SPRI NILs, thus any gene conferring disease escape linked to Psp3071 in the FLLO material is likely also to have been associated with it in the SPRI material.

3) The predicted effect for SPRI is smaller than for FLLO and the trials were not large enough to detect them

The data from Ireland showed that when the difference was present in both lines they were of a similar magnitude.

4) The disease escape is affected by environmental factors, and does so to a different extent in the different genotypes.

This is the most likely explanation based on our data. It has been shown that there are GxE effects on physiology and development affected by the 6A locus. Environmental differences many therefore have led to variation in disease escape between the Spark and Rialto alleles in Ireland but not Norfolk.

5) There is no disease escape effect, and the differences are explained by a resistance effect with strong genotype by environment interactions.

This explanation always needs to be considered and the variation between the genotypes and sites indicates that G x E effects was important. However the lack of differences in lower leaf layers and the experiments in Chapter 6 support the disease escape hypothesis and disease escape is more likely to be subject to strong G x E effects than resistance genes.

Despite there not being significant differences between the Spark and Rialto alleles in Norfolk overall, there were significant increases in STB the SR-6 and SR-21 recombinants in the S5 and S4 leaf ranges. These recombinant lines are the same as Spark at psp3071, but have Rialto further along in the 6A chromosome. The increase in STB levels in these recombinant lines does not occur in S1-S3. The lack of an effect in

the lower canopy supports the hypothesis that the STB difference in the recombinant population was caused by disease escape rather than resistance.

The SPRI plants were taller than FLLO plants (Figure 4.3.1), and increased plant height reduces levels of STB (Arraiano et al., 2009, Van Beuningen and Kohli, 1990). The S5 measurements showed the SPRI plants had less STB on their flag leaves (Figure 5.3.2). However the max STB height data showed that STB spread to a lower point on the canopy, as opposed to spreading to the same height, with the flag leaves above it. This implies that a factor other than height caused the lower STB spread in SPRI than FLLO. This may be due to differences in other developments traits affecting disease escape or to partial resistance to STB reducing disease spread by limiting production of inoculum. The max STB height data is also relevant when considering the difference between the FLLO families. Family 16 had significantly higher levels of STB in the field trials than family 24. It also had higher levels of STB in the inoculated experiments (Chapter 3) indicating a difference in susceptibility between the families. However the max STB height data showed that the difference between the families was not just the results of a difference in the amount of infection on individual leaves, but that disease spread upwards faster in family 16. This greater spread in family 16 led to increased significance of the factor "family" on STB in each successive height range. This can be seen in the V.R values for family in Table 5.3.1. This may relate to differences in spore production between the families, or to alternative disease escape traits.

The max STB height data shows that the NILs did not significantly differ in the height that spores of the fungus reached on plants of different genotypes (Figure 5.3.4) while the adult plant inoculation data shows that the lines did not significantly differ in resistance to STB (Chapter 3). Yet there are significant differences caused by the 6A locus in the amount of STB in the upper canopy of FLLO field trials and of SPRI in Ireland. If spores of *Z.tritici* arrived on the upper leaves sooner in NILs with the Longbow allele than Flame, this would provide a greater amount of time for horizontal spread within the leaf layer. However there is not a Timepoint x Allele interaction for the Max STB height data. This lack of interaction between the effects of Timepoint and Allele on Max STB height indicated that the disease moved upward on plants of the contrasting alleles at the same time. If the timing of the disease spread was the same

between the alleles, the source of the greater STB scores for plants with the Longbow allele is implied to be due to more spores spreading upwards than in the canopy of plants with the Flame allele when heavy rainfall occurs.

Summary

Chapter 3 showed that the 6A alleles caused no significant differences in STB when leaves were directly inoculated. This was found in seedling tests (Figure 3.3.3 & 3.3.5) and in adult plants (Figure 3.3.8, & 3.3.10). Chapter 5 showed that there can be differences in STB levels on the flag leaf in the field. The presence of differences in STB in the upper canopy under natural infection but not when plants were inoculated supports the hypothesis that differences in STB between the 6A NILs were caused by differences in disease escape rather than resistance. These field differences occurred in FLLO in all trials but only in the Irish trial for SPRI. Chapter 4 identified some candidate escape traits that may relate to the difference. This is tested further experimentally in Chapter 6.

6 Epidemiology experiments on the effect of the 6A locus on splash-borne dispersal of *Zymoseptoria tritici*

6.1 Introduction

The spread of disease in crops is often studied by epidemiological modelling (Grassly and Fraser, 2008). This can be done on various different scales, from studying how virulent strains move between countries (Kolmer, 2005), to spread within a field or on an individual plant (Pielaat et al., 2002). Epidemiological modelling needs to take into account the known features of the disease being studied. Key traits used in epidemiological models include the mechanism of disease spread, the pathogen's lifecycle and the effect of plant defences.

The primary infection of wheat seedlings with STB can occur from infected seeds and via spread from crop debris or alternative hosts (Wenham, 1959, Brokenshire, 1975). However the most common method of primary infection is from airborne *Z.tritici* ascospores released from the sexual stage of the fungus (Shaw and Royle, 1989b, Sanderson, 1972). The subsequent spread of disease within a crop is caused by splash borne dispersal of pycnidiospores (Bahat et al., 1980, Sanderson and Hampton, 1978, Shaw and Royle, 1993). As a mechanism for causing disease spread, rain splash allows for transfer of spores horizontally for a relatively small distance even in windy conditions, making it unlikely to spread disease between fields (Brennan et al., 1985). Vertical movement of spores depends on the rain intensity and crop canopy, whilst dispersal is most likely over short distances, rain splash has been shown to lead to vertical spore movement of over 1m (Faulkner and Colhoun, 1976). Knowing that the above methods affect how STB is spread allows more accurate modelling of how disease progression in the field occurs and predictions to be made about how escape traits may be operating.

Studies into the spread of STB can be performed by analysing field data for significant relationships between traits and the scored STB in the canopy. These field association tests can show the relationship with environmental factors (Polley and Thomas, 1991)

or with physiological and developmental traits (Arraiano et al., 2009). Studying disease escape in non-field experiments is less common, with initial experimental studies into the effect of rain splash being focused on the physical dynamics of the rain splash (Fitt et al., 1989, Walklate, 1989). Experimental tests of the extent of disease spread have been performed on various splash borne diseases (Madden and Ellis, 1990, Ntahimpera et al., 1998, Soleimani et al., 1996), but there have been few experimental tests of the epidemiology of STB. The effect on STB disease escape from altering the spacing in the canopy with PGR's was studied experimentally with varied levels of watering by Lovell et al (2004). This type of experiment can be adapted to test whether or not the morphological and developmental differences reported in Chapter 4 can cause variation in disease escape in an environment with controlled precipitation.

In Chapter 4 the physiological traits of leaf area and leaf senescence were identified as being different between the NILs. These traits may cause differences in the levels of STB on upper leaves by influencing spore transmission. In Chapter 5, significant differences in field levels of STB were found on the flag leaves (S5) of the crop but not in the lower leaves (S1-S4). This difference in flag leaf STB was not found when the leaves were inoculated directly in Chapter 3. The presence of STB differences in the field trials (Figure 5.3.2) compared to adult plant tests, AP1 and AP2 (Figure 3.3.8 & 3.3.10) may be due to natural infection allowing escape differences that would not occur when leaves are directly inoculated. Alternatively, the change in environment may be the cause of the different effect of the 6A locus on FLLO flag leaf STB. This can be tested by either directly inoculating the flag leaves in field trials or by setting up plastic glasshouse experiments where the Z*.tritici* spores have to reach the flag leaf via rain splash. This chapter shows experiments performed for the second of these two options.

The initial infection with STB in field conditions will usually occur in the winter, whilst the plants are still seedlings. To align with the lifecycle of STB found in the field, the experimental tests on the role of disease escape have to be inoculated before stem extension. This means that any disease levels on the flag leaves are affected by both the resistance of the leaf and the degree of disease escape. Transfer of the spores to

the top of the plant is achieved by heavy watering of the plants from above. The contrast between the basally inoculated plants and the flag leaf inoculated plants should give an experimental way of assessing any differences in disease escape affected by the 6A alleles in the NILs.

Chapter aims

In this chapter I aim to establish experimentally if disease escape could be causing differences in levels of STB between the NILs.

1) To establish a system that allows hypotheses about the epidemiological role of splash-borne pathogens spores to be tested experimentally.

2) Does the 6A locus significantly affect STB levels in the Plastic glasshouse disease escape experiments?

3) Do the levels of STB on the lines in epidemiology experiments support the hypothesis that the 6A locus alters STB by affecting disease escape?

6.2 Materials and methods

Materials

In this chapter, the three experiments performed to test for the disease escape differences are referred to as Esc0, Esc1 and Esc2 and were performed in 2013, 2014 and 2015 respectively. The lines of wheat used in these experiments were the 6A FLLO and SPRI NILs detailed in Chapter 2.2 and Chapter 2.3. Esc1 and Esc2 had replicates of the main 15 NILs in two blocks, with the ten additional Spark x Rialto recombinants being included once (Table 2.2.2 and Table 2.3.2). The randomisation and blocking of the experimental set ups is described in the Chapter 2.4 (Figure 2.4.1, Figure 2.4.2 and Figure 2.4.5). The fungal isolates used are part of the Brown lab's collection of *Z. tritici*. The lines selected for testing are English and Dutch isolates that are acceptable for use in a glasshouse environment and are the same ones as used the equivalent adult plant experiments (Table 3.2.4).

Methods

EXP	Location	Isolates / Block	Inoculation Date	Plants per Allele	Scoring range	Watering
Esc0	Glasshouse 53 2013	JIC040 CHC3 IPO94269	May 3rd 2013	24 per watering type	77 Dpi-82 Dpi	3 types of manual application
Esc1	Plastic glasshouse 2014	JIC040 CHC3	April 2nd 2014	64	64 Dpi-82 Dpi	Timed from Ceiling
Esc2	Plastic glasshouse 2015	CHC3 CHC3	April 2nd 2015	64	63 Dpi-84Dpi	Timed from Ceiling

Table 6.2.1: Set up of escape test experiments. Dpi is number of days post infection with *Z.tritici.* Each block is infected with a different isolate, apart from Esc2 where both blocks were infected with CHC3.

The concept behind the experimental design was to devise a method of establishing the degree of disease escape from splash borne pathogens using small groups of plants. These experiments were not based on any pre-existing protocols hence the

need for refinement of the method between the different iterations. To separate the disease escape effect on disease levels from the resistance response, appropriate controls needed to be used. These experiments needed to be performed in conjunction with standard tests of resistance to allow for the correct comparisons to be drawn. To this end, Esc0 and AP1 were performed in the same conditions and environment as were Esc1 and AP2.

Preliminary experiment (Esc0)

Esc0 was performed in glasshouse 54 in 2013, which is set up to grow the plants at ground level like a poly-tunnel. The plants were grown in groups of 16 plants, with a 4 x 4 arrangement being selected to allow for ignoring the edge effect by taking measurements on the central four plants (Figure 2.4.4). These plots of 16 plants were organised into three blocks for testing the three different isolates (Figure 2.4.1). Each block contains 16 plots; four contained BC2 Spark x Rialto lines (118, 119, 116, 117) whereas the other 12 are repeats of four different Flame x Longbow NILs (16A3, 16B8, 24C15 & 24D16) under three sets of watering conditions.

All plants within the experiments were watered from below via pipes running through water retaining matting. Sufficient watering was supplied though the matting twice a day to maintain healthy plant growth. Three times a week, 0.5L of additional water was added to the plots in three different ways to test the role watering plays in the disease escape process. Watering only from below was used as a negative control; to test that rain splash is the cause of disease spread. A hose with a sprinkler attachment was used to provide overhead watering with large droplets of water, hereafter referred to as “Sprinkler”. Tests with the sprinkler set up beforehand showed that watering for 4 seconds 130cm above the plots provided 0.5 L of water to the pots. The third type of watering “Misting” used a handheld pressured sprayer. This was performed for 30 seconds to achieve the same volume of water.

In the field, plants involved in spore transmission will be surrounded by plants of the same variety. To prevent the different NILs being tested from affecting neighbouring varieties, the plots are kept separate by transparent sheets of PET (1x750x500mm), forming a grid surrounding the plots on all sides.

Figure 6.2.1: Example of a plot set up for Escape trials.

The plants were grown within these blocks from February 2013 to July 2013. Bulked fungal inoculum was applied using the backpack sprayer (Chapter 2.5) at a walking speed fast enough for the spray to be applied evenly but slow enough to avoid turbulence around the plastic barriers. Each of the three blocks was infected with a different isolate and the sprayer was washed between them. This infection process has to be performed prior to the emergence of the upper leaf layers, thus ensuring that any symptoms that develop on the flag leaf have been subject to disease escape. For Exp0 the plants were infected on the third of May. After the inoculation the plots were left for the disease to develop, whilst also having the additional watering being provided every 2-3 days to aid the spread of the fungus. The scoring of the Escape tests was performed with the same methodology and scale as the adult plant experiments (Chapter 2.5). The top three leaves of all of the tillers of the central four plants were assessed for levels of STB, to maximise the number of data points whilst minimising edge effects from the barriers. The scoring of escape experiments can only be performed within a small timeframe because the first score will involve moving the plants involved, with frequent scores affecting spore movement (Lovell et al., 1997). Therefore all scoring in any given plot, has to be completed before any symptoms could have developed from the first score. Given the long latent period of STB, this time period was taken as approximately two weeks.

Esc 1 & Esc2

Esc0 was performed as a preliminary experiment to test the principles of designing this kind of experiment. Infection and scoring of the plots was performed using the same method as Esc 0. However the watering system and lines used were altered for the subsequent experiments.

Individually watering the plots in three different randomised methods was time consuming. In addition manual watering with the different implements raises the question of procedural error, as despite being done to the same timings and protocol, it is very feasible that different individual plants didn't always receive the same amount of water. To fix this issue, overhead watering was done mechanically in the main experiments. This was achieved by laying piping into the roof of a plastic glasshouse, with spray nozzles affixed at set intervals. This system was attached to a timer, so that heavy overhead watering could be applied for a set time period twice a day. The plots were arranged on either side of a central line with the pipes above it (Figure 2.4.3). This system created less work for the operator of the experiment and allowed a greater volume of water to be applied at minimal extra effort. The timer was initially set to provide 2 minutes of water twice a day, but was adjusted based on the plants requirements. This system also had to be inactive on either side of the inoculation so that it wasn't diluted and washed off the leaf. Watering was provided via matting during this time. In the preliminary experiment, plants with no overhead watering were used as a negative control. This was not retained as it would require a mechanism for impeding the ceiling watering, and the space would be better used to having additional test plots. The lines used in Esc1 and 2 were the main 15 used for field trials, (Figure 2.2.2). In addition, the 10 SPRI recombinant lines were also available in sufficient amounts by 2014, and they were included in one of the two blocks (Figure 2.3.2). There was only enough space for one plot of each individual line for each isolate, however each plot produced a lot of data due to the number of tillers measured, and each allele at the 6A locus has 8 plots in total. The plot layouts of the experiments were generated by randomising the lines with a random number generator, to distribute the NILs fairly (Figure 2.4.2 &2.4.5).

6.3 Results

Esc0 Results

The Esc0 experiment was a test of the proof of concept for experiments on disease escape. Watering from below led to no STB in the upper canopy, supporting the requirement of splashing water for spore transport. The disease incidence was low in the experiment overall, with around 95% of the flag leaves and 75 % of the second leaves having no STB symptoms (Figure 6.3.1).

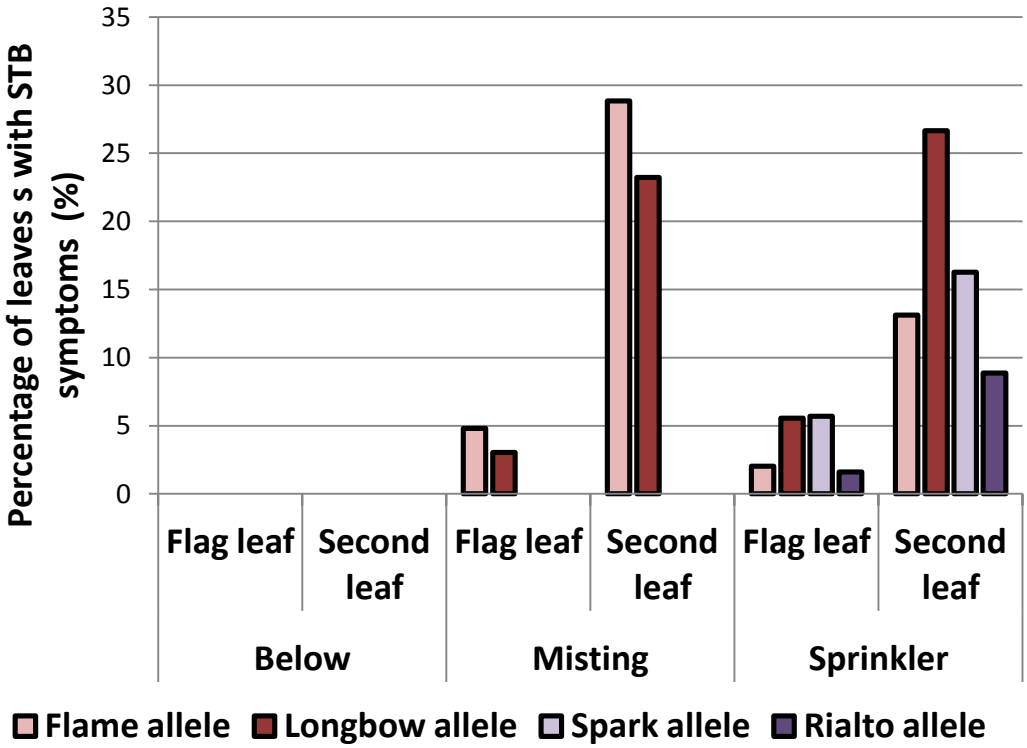

Figure 6.3.1: Percentage incidence of STB symptoms in Esc0. The data given is the percentage of sampled leaves with symptoms out of the total number of leaves sampled. This is split by the leaf layer tested and the three different types of watering, watering from below only and overhead watering with a mister and a sprinkler.

There was no significant difference between the two overhead watering methods; with the misting method leading to similar levels of STB to the watering with the sprinkle. This implies that similar amounts of spore movement occurred, however the relative humidity differences may have also contributed. The flag leaves got significantly lower levels of STB than the second leaf in both the misting and sprinkler watering

treatments, consistent with the movement of spores up the plant. There was variation in the amount of disease caused by the different isolates, with the English isolate CHC3 leading to a higher level of STB than the other two.

Logit adjusted % AUDPC data was analysed with the model (**Isolate+Watering+Leaf+Family) *Allele +Plot+Plant position**. The allele at the 6A locus did not significantly affect the amount of Septoria symptoms on the upper leaves of the plant. Whilst there was not a significant difference between the alleles, the trend is towards greater amounts of STB on the lines with the Longbow allele. Despite the Longbow and Rialto alleles being expected to both show increases in STB, in the Spark x Rialto NILs there was less STB on plants with the Rialto allele. However, the data from Esc0 is not considered to be reliable due to it being a preliminary experiment with very low scores and very high number of leaves that had zero STB symptoms (Figure 6.3.1).

Esc1 results

The changes made to the protocol for Esc1 led to greater disease spread than Esc0. Compared to manually watering the plants, the automatic timed system was able to supply a far greater amount of water, aiding the disease spread. However the experiment did suffer from contamination from other pathogens. The plots became infected with both powdery mildew (*Blumeria graminis* f.sp. *tritici*) and yellow rust (*Puccinia striiformis* f.sp. *tritici*). The growth of yellow rust in plastic glasshouse conditions was unusual and may reflect the growing prominence of new aggressive races of it in the UK (Hubbard et al., 2015, Hovmøller et al., 2015).

Whilst the 6A locus did not significantly affect the other pathogens, the background of the plants had a large effect. The Flame x Longbow plants got low levels of mildew and high levels of yellow rust, whereas the Spark x Rialto plants showed the opposite effect (Figure 6.3.2).

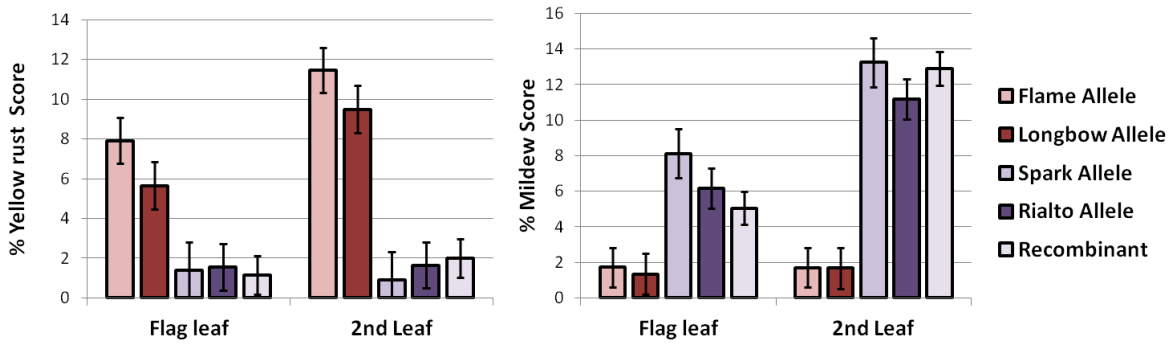

Figure 6.3.2: Predicted means of yellow rust and powdery mildew scores for the 2014 disease escape experiment (Esc1). The means were generated for the contrasting 6A alleles in the Flame x Longbow and Spark x Rialto NILs. Error bars are +/-1 standard error of the mean.

The STB scores in Esc1 showed a far greater level of disease than Esc0, with both incidence and severity of the disease increasing. The two blocks in the experiment were infected with the isolates JIC040 and CHC3 at the base of the plant. The block infected with JIC040 had very low values for %AUDPC across the two time points. JIC040 had also led to lower levels of STB than CHC3 in Esc0. However, JIC040 was no less virulent than CHC3 on these lines in AP1 or AP2 (Figure 3.3.9 & Figure 3.3.11). This indicates that there may have been an aspect of the escape experiment that caused the difference between the isolates.

That the isolates are infected by being sprayed over a large block means that the isolate factor also includes positional information, which may be responsible for the effect. In addition the yellow rust contaminated the two isolate blocks differently, with the JIC040 block having a greater amount of yellow rust. Leaves covered in dead yellow rust were very difficult to score and may have led to underestimation of STB symptoms as scoring was performed erring on the side of calling ambiguous symptoms as not septoria.

The upper leaves of the plants had different levels of STB (Figure 6.3.3). Leaves with the Psp3071-167 maker at the 6A locus, (Flame & Spark) had less STB pycnidia on them than those with the alternative alleles. This difference was greater between 161 and 167 (Longbow and Flame alleles respectively), than between 152 and 167 (Rialto and

Spark alleles respectively). This fitted with the initial field data on STB levels from the association genetics study (Figure 2.1.1). The difference in disease levels was significant in both the Flame x Longbow population and the Spark x Rialto population (Table 6.3.1 and Table 6.3.2). However in both populations, the low levels of STB in the block infected with the JIC040 isolate was an important factor. It explained the interaction between isolate and allele, which was significant in the SPRI background and almost significant in the FLLO background. The lower disease levels made any differences between the alleles less distinct compared to the block infected with CHC3, which shows differences clearly (Figure 6.3.3). When scoring was performed a large proportion of the third leaves were too senescent to score accurately, so only the second leaf and flag leaf were included in the analysis.

Esc1: Analysis of Variance (FLLO)			
Variate: Logit %AUDPC			
Model: (Isolate+Leaf+Family)*Allele +Plant position+ Plot			
	d.f	v.r	F pr
Isolate	1	100.85	<.001
Leaf	1	0.67	0.41
Family	1	0	0.97
Allele	1	11.54	<.001
Isolate X Allele	1	3.5	0.06
Leaf X Allele	1	1.95	0.17
Family X Allele	1	0.65	0.42
Position	3	1.89	0.12
Plot	10	11.78	<.001
Residual	106		

Table 6.3.1: Analysis of variance of STB in the Flame x Longbow plots of the 2014 disease escape experiment (Esc1). The analysis was performed on logit adjusted % AUDPC scores with the model **(Isolate +Leaf +Family) *Allele +Plant position+ Plot.** Abbreviations are as described in Table 3.3.1. Logit transformation as described in Table 3.3.2.

Esc1: Analysis of Variance (SPRI)			
Variate: Logit %AUDPC			
Model: (Isolate+Leaf+Family)*Allele +Plant position+ Plot			
	d.f.	v.r.	F pr.
Isolate	1	68.62	<.001
Leaf	1	3.89	0.052
Family	1	0.69	0.41
Allele	1	11.07	<.001
Isolate X Allele	1	5.8	0.02
Leaf X Allele	1	5.83	0.02
Family X Allele	1	0.18	0.67
Position	3	0.24	0.92
Plot	8	1.15	0.34
Residual	92		

Table 6.3.2: Analysis of variance STB in the Spark x Rialto plots of Esc1. The analysis was performed on logit adjusted % AUDPC scores with the model **(Isolate+Leaf+Family) *Allele +Plant position+ Plot**. Abbreviations are as described in Table 3.3.1. Logit transformation as described in Table 3.3.2.

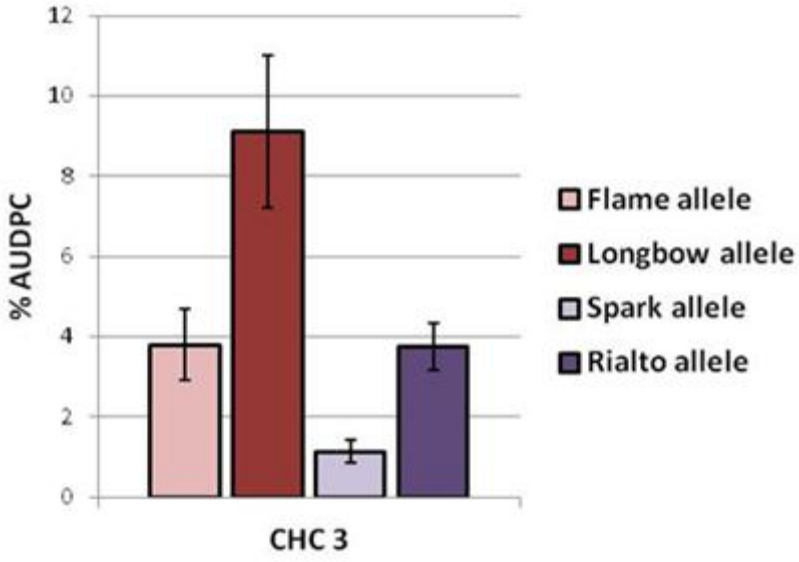

Figure 6.3.3: Average scores of STB from the upper canopy of the 2014 disease escape experiment (Esc1). The data is separated into which parent contributed the allele at the 6A locus in the two populations Flame x Longbow (FLLO) and Spark x Rialto (SPRI). Values shown are predicted means from the model in Table 6.3.1 & 6.3.2. The average includes data points from both the flag and second leaf from both isolates tested. Error bars are +/-1 standard error of the mean.

Esc 2 Results

In the 2015 plastic glasshouse disease escape test (Esc 2), there was a similar level of STB in the upper canopy of the plants to the previous year's experiment (Esc 1). The disease levels in the upper canopy were significantly affected by the different 6A alleles in the Flame x Longbow NILS (Table 6.3.3), and there was some evidence for a difference between the Spark and Rialto alleles (Table 6.3.4). However the direction of the trend in Flame x Longbow is the reverse of the effect seen in the field trials (Figure 5.3.2) and Esc1 (Figure 6.3.3), with greater levels of STB in lines with the Flame allele (Figure 6.3.4). Leaf is highly significant for both backgrounds with levels of STB decreasing at higher leaf layers (Figure 6.3.5). The two different Flame x Longbow families, also showed large differences, with family 16 leading to much higher disease levels than 24, although as with the field trial data, this effect did not interact with the Allele effect.

Esc2: Analysis of Variance (FLLO)			
Variate: Logit % AUDPC			
Model: (Block+Leaf+Family)*Allele+Position+Plot			
	d.f.	v.r.	F pr.
Block	1	8.97	0.003
Leaf	2	257.96	<.001
Family	1	598.96	<.001
Allele	1	33.69	<.001
Block x Allele	1	6.64	0.011
Leaf x Allele	2	0.33	0.718
Family x Allele	1	0.65	0.42
Position	3	2.72	0.046
Plot	10	3.24	<.001
Residual	163		

Table 6.3.3: Analysis of variance of STB in the Flame x Longbow plots of the 2015 disease escape experiment (Esc2). The analysis was performed on logit adjusted % AUDPC scores with the model **(Block+Leaf+Family) *Allele +Plant position+ Plot**. Abbreviations are as described in Table 3.3.1. Logit transformation as described in Table 3.3.2.

Esc2: Analysis of Variance (FLLO)			
Variate: Logit % AUDPC			
Model:(Block+Leaf+Family)*Allele+Position+Plot			
	d.f.	v.r.	F pr.
Block	1	18.87	<.001
Leaf	2	210.31	<.001
Family	1	0.77	0.381
Allele	1	3.43	0.066
Block x Allele	1	18.03	<.001
Leaf x Allele	2	0.86	0.426
Family x Allele	1	2.01	0.158
Position	3	1.56	0.202
Plot	8	6.87	<.001
Residual	147		

Table 6.3.4: Analysis of variance of STB in the Spark x Rialto plots of the 2015 disease escape experiment (Esc2). The analysis was performed on logit adjusted % AUDPC scores with the model **(Block+Leaf+Family) *Allele +Plant position+ Plot**. Abbreviations are as described in Table 3.3.1. Logit transformation as described in Table 3.3.2.

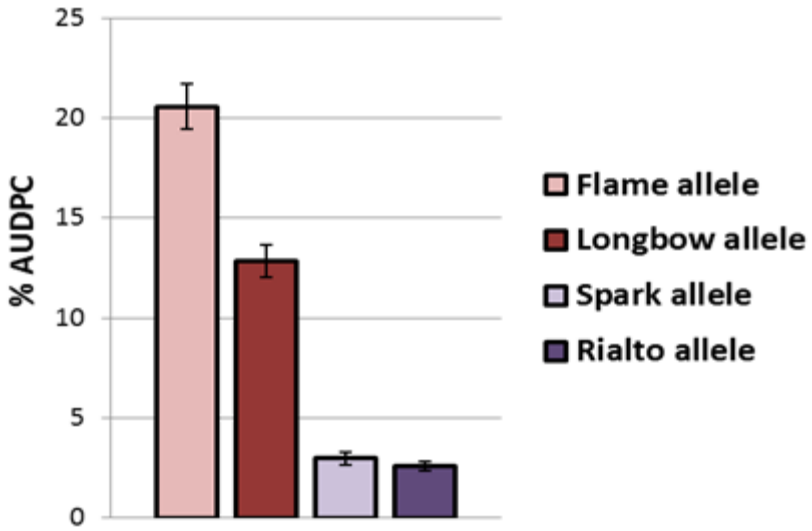

Figure 6.3.4: Average scores of STB from the upper canopy of the 2015 disease escape experiment (Esc2). The data is separated into which parent contributed the allele at the 6A locus in the two populations Flame x Longbow (FLLO) and Spark x Rialto (SPRI). Values shown are predicted means from the model in Table 6.3.3 & 6.3.4. The average includes data points from all three leaf layers. Error bars are +/-1 standard error of the mean.

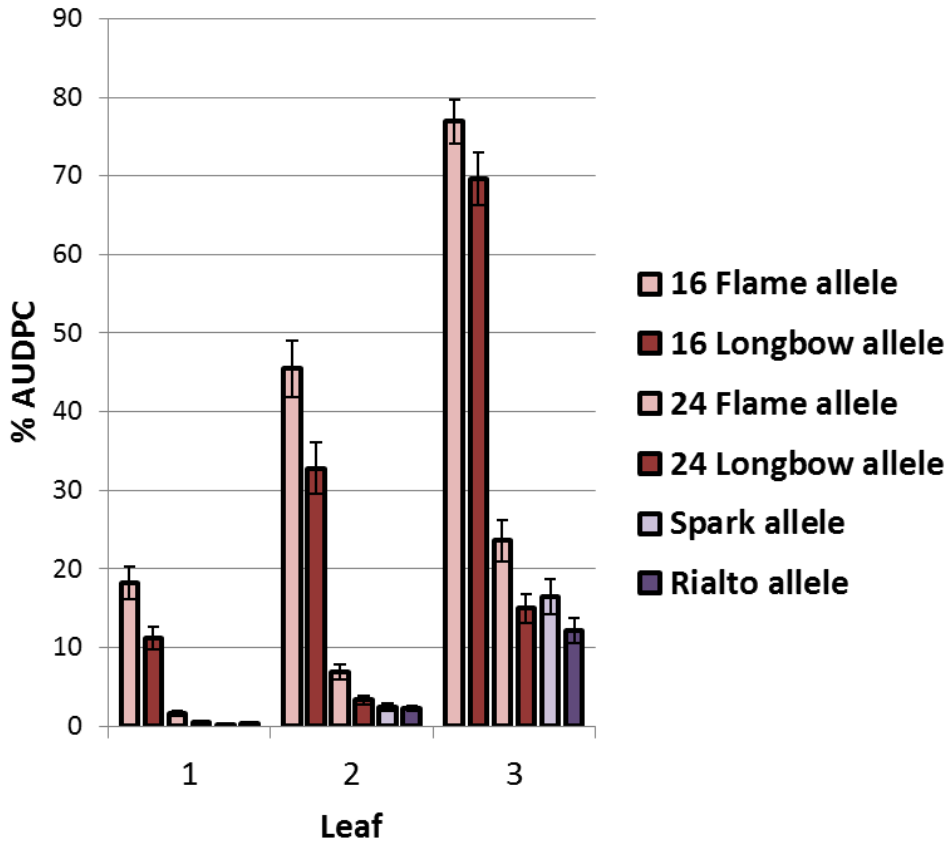

Figure 6.3.5 Average scores of STB from the individual leaf layers of the 2015 disease escape experiment (Esc2). The data is separated into which parent contributed the allele at the 6A locus in the two Flame x Longbow (FLLO) families and Spark x Rialto (SPRI). Values shown are predicted means from the model in Table 6.3.3 & 6.3.4. Error bars are +/-1 standard error of the mean.

Both Esc1 and Esc2 also included Spark X Rialto recombinant lines. There was a significant difference between the different recombinants across the region in both years. Both experiments had one genotype that had a far higher level of STB than the others; these were SR-21 and SR-6 for Esc1 and Esc2 respectively (Figure 6.3.7 and 6.3.8).

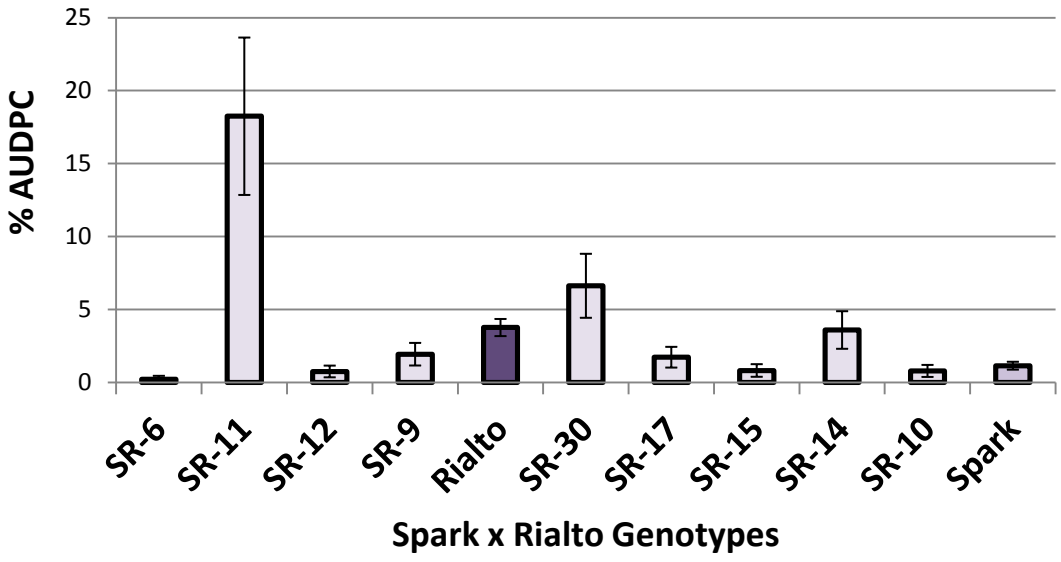

Figure 6.3.7: Average scores of STB from the upper canopy of SPRI recombinants in the 2014 disease escape experiment (Esc1). The data is separated into the different genotypes found across the 6A region in the Spark x Rialto (SPRI) population. Values shown are predicted means from the model **Leaf*Allele+Line+Plant position+ Plot**. Error bars are +/-1 standard error of the mean.

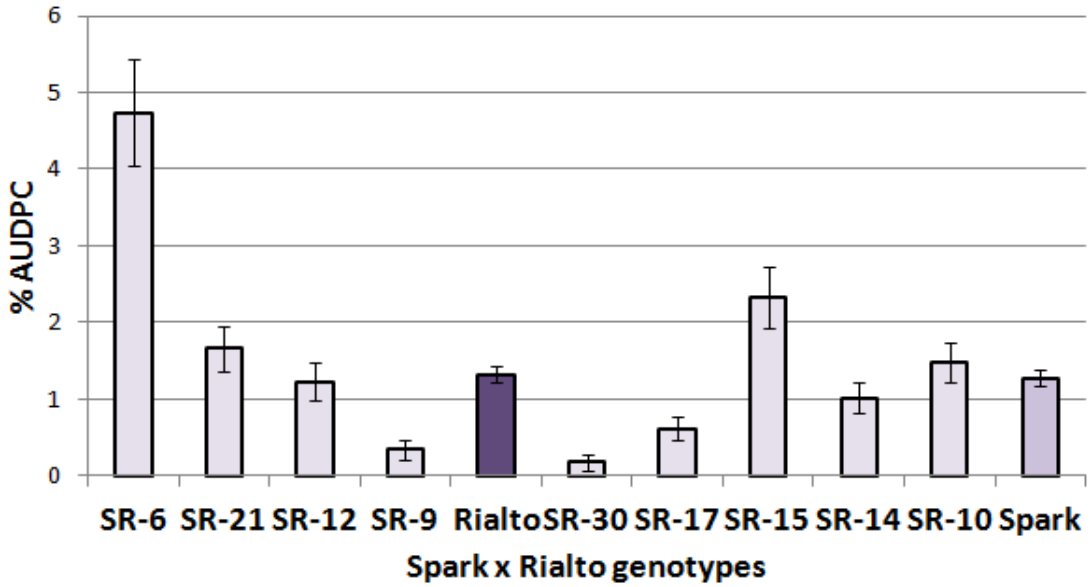

Figure 6.3.8 Average scores of STB from the upper canopy of SPRI recombinants in the 2014 disease escape experiment (Esc2). The data is separated into the different genotypes found across the 6A region in the Spark x Rialto (SPRI) population. Values shown are predicted means from the model **Leaf*Allele +Line+Plant position+ Plot**. Error bars are +/-1 standard error of the mean.

6.4 Discussion

Whilst there are many studies supporting the spread of STB by splashy rainfall (Polley and Thomas, 1991, Shaw and Royle, 1993), spread via leaf contact has also been observed when newly emerging leaf layers are at a similar height to existing lesions. Lovell et al (2004) tested this experimentally by allowing trays of wheat seedlings to be naturally infected and subjecting them to differing watering treatments. The greatest levels of STB were observed under conditions with light rainfall, but STB still developed on the upper leaves of the plant under misting, misting with wind and at a lower level with the control without watering. In contrast, Esc0 only developed symptoms when overhead watering was applied. When water was only provided at the base of the crop, STB symptoms didn't develop above the initial infection height. This difference is presumably because Lovell used plants with a short stature that were naturally infected as seedlings and thus had lesions already developed when the leaf emergence occurred. In our disease escape experiments, infection was performed closer to the time of stem extension. This removed the potential for contact transfer prior to the plant growth, leading to all spore movement being due to splash borne transfer. The importance of splashy rain in the transfer of septoria is also supported by the increased amount of infection in Esc1, compared to Esc0, when overhead watering was performed with a greater volume of water more regularly.

The main hypothesis for the experiments reported here was that, if physiological or developmental differences between the 6A NILs cause differences in disease escape, the upper leaves of the plant will show differences in STB levels, when spores of *Z.tritici* are spread though the canopy by artificial rainfall. The different alleles at the 6A QTL significantly affected the extent of STB scored on the upper leaves, in Esc1 and Esc2. The scores on the leaves in these experiments were a combination of the effect of disease escape restricting spore contact with the leaf and the resistance of the leaves to infection by *Z. tritici* spores. Flag leaf inoculation tests, performed in the same environment, showed that the NIL pairs had no difference in their resistance to the disease (Chapter 3), this indicates that the differences between the NILs was due to differences in disease escape.

Esc 1 shows that the Longbow and Rialto were alleles associated with increased STB levels, as predicted by the association genetics (Figure 2.1.1) and found in the field trials (Figure 5.3.5). Whereas Esc2 shows the reverse effect, with the Flame allele causing significantly higher STB levels than Longbow, and whilst not quite significant, the trend was also reversed in SPRI (Figure 6.3.4). This reversal in the direction of effect of the alleles on disease escape needs an explanation. The working hypothesis for the differences in STB levels is that the 6A locus affects physiological and developmental traits, which subsequently alter disease escape. In Chapter 4, several candidate traits for affecting disease escape had GxE effects that differed between the 2014 and 2015 plastic glasshouse experiments. Leaf area, SPAD readings and height to the ear base all showed a shift from being increased by the Longbow and Rialto alleles towards being increased by the Flame and Spark allele (Table 6.4.1). Thus despite the disease trend in Esc2 reversing from the one shown in Esc1 and by the field data, the hypothesis of physiological and developmental traits causing the STB effect, still holds because the genetic effect on the physiological traits also reversed direction in this environment. Why that happened is not known.

	STB in Esc tests	Leaf area	Leaf greenness (SPAD)	Height (Ear base)
Tunnel 2014 FLLO	Longbow ***	Longbow *	NS	Longbow **
Tunnel 2015 FLLO	Flame ***	NS	Flame **	Flame *
Tunnel 2014 SPRI	Rialto ***	Rialto *	Rialto +	Rialto ***
Tunnel 2015 SPRI	Spark +	Spark +	Rialto *	NS

Table 6.4.1: Physiological and developmental traits compared to STB data from escape tests in 2014 and 2015. The 6A allele that is associated with the higher values in each trait is listed, alongside of the significance of the interaction. NS is used for traits that were not significant in that experiment.

The data showed larger amounts of STB in FLLO than SPRI, this difference was far larger than that seen in the inoculation tests and was presumably caused by the addition of the disease escape effect of the SPRI population's greater height to the increased background resistance of the SPRI material. It would be expected that leaves further from the initial source of inoculums would have less STB, so the decreasing levels of STB at each leaf layer in Esc 2 fits with this prediction (Figure 6.3.5). The

greater levels of STB in plants from the Flame x Longbow family 16 aligns with the previously collected data from the field trials, where significantly higher STB levels and increased disease spread were found for family 16 (Chapter 5).No effect of family or leaf, however, was detected in Esc1.

The SPRI recombinant data from Esc 1 and Esc 2 showed a high level of variability between genotypes (Figure 6.3.7 and 6.3.8). The recombinant data from the field trial in 2015 produced similar results (Figure 5.3.3). As neither set up showed large differences between Spark and Rialto the genotypes breaking up this area could not narrow down the region further. However, in the field trial SR6 and SR21 showed higher disease levels than either Spark or Rialto, indicating that there may be a gene that increases disease spread in these lines. This is supported by the Esc 1 and Esc 2 data, as in each of them; one of these recombinants got a very large amount of STB.

Phenotypic variation between lines has been shown to lead to significant differences in disease spread between lines, for example variation in height and leaf spacing in (Bahat et al., 1980). However the strong GxE interaction on disease escape, allowing for the reversal of the effect, seems to be unprecedented in the literature. This raises questions about the cause of the effect reversal and its implications for disease management in environments where it occurs. These escape experiments are also a reversal of the usual direction of this type of study. It used lines that were predicted to be differing for their amount of disease escape and tried to identify the traits that cause it, as opposed to looking by adjusting specific trait, like height (Bahat et al., 1980) or sowing rate (Baccar et al., 2011) and trying to establish if this leads to escape differences.

These experiments were devised to develop a system that was capable of testing for disease escape effects in a plastic glasshouse. The difference between the response to direct inoculation of the flag leaves and the Esc1&2 data supports that these lines have a difference in disease escape.

Disease escape is highly related to environmental factors, thus the ability to identify differences in disease escape in controlled conditions is useful for studying how it operates. The method developed in this chapter could be used for studying how

disease escape relates to other traits and other diseases, though would ideally be scaled up to allow for testing a larger amount of plots at once. It would also be of interest to perform this kind of test separating the plots using discard areas as opposed to barriers, allowing wind to move the plants more freely, as this should be a more accurate simulation of what would occur in the field. The method is also suitable for comparing NILs that differ in a known trait, to see if this leads to disease differences. The work on the Rht NILs is an example of its use at testing the role that specific genes have on disease escape. The method could also be adjusted to sow and infect the plants earlier in the year, This would have the advantage of allowing greater inoculums build up and contact transfer when emergent leaves are closer to lesions as in Lovell et al (2004). This would more closely replicate field conditions but by increasing the likelihood of contact transferral of STB, would prevent any differences found from being identified as clearly interacting with rain splash.

Summary

Despite the fact that when the flag leaves of the 6A NILs were inoculated with *Z.tritici* they did not show significant differences in levels of STB (Chapter 3), the upper leaves of plants inoculated at the base did show significant differences in STB affected by the 6A locus. These differences occurred under conditions of heavy rainfall generated by pipes in the ceiling of a Plastic glasshouse. The direction of the effect of the 6A alleles reversed between experiment Esc1 and Esc2 but the hypothesis of physiological and developmental traits causing different levels of disease escape was supported by several physiological traits also reversing direction in this environment.

7 General discussion

7.1 Introduction

The project started with the finding that the marker psp3071 on chromosome 6A was associated with both different levels of Septoria tritici blotch in the field (Arraiano and Brown, 2016) and increased yield (Snape et al., 2007). The mechanism behind either effect was unknown and it was speculated that they may be caused by the same gene, in a pleiotropic effect. In this thesis, the STB differences have been studied in near isogenic lines that differ in their alleles at the 6A region. The data supports the hypothesis that developmental traits controlled by this region lead to differences in STB levels via changes in disease escape. Whilst significant differences in both STB levels and yield were found associated with the 6A locus, it is not yet clear whether or not the effects are pleiotropic.

Summary of results

Chapter 3 tested the response of the NILs to direct inoculation with different *Z.tritici* isolates. The 6A locus was not associated with significant differences in the resistance of the plants to STB. This raised the question of whether or not the NILs would show differences in field trials, as predicted by the association genetics study. The field trial experiments in Chapter 5 showed significant differences in STB on the flag leaf for all FLLO material and for SPRI lines in the Irish trial. Having established that differences in STB levels occur between the 6A alleles in the field but not in the glasshouse inoculations, differences in disease escape between the alleles seems to be a likely cause of the effect.

This hypothesis was tested by the disease escape tests in Chapter 6. Significant differences in levels of STB in the upper canopy were found between lines with the different alleles under the same conditions as the tests in Chapter 3 plus earlier infection and overhead watering. Several physiological and developmental traits were measured on the NILs in Chapter 4. Some of these traits showed significant differences between lines with the different 6A alleles. These can be considered as candidate traits for being the cause of the disease escape difference.

7.2 Which candidate trait best fits the disease results?

To identify which candidate trait is most likely to be causing the disease escape effect, the results from the experiments can be compared. An escape trait should show the same direction of difference for the FLLO and SPRI backgrounds with Flame and Spark and Longbow and Rialto causing the same effects respectively. It would also be expected that the differences in FLLO would be greater than those found in SPRI. In addition, if a trait is causing changes in disease escape, its genotype-by-environment (G X E) effects should be reflected in the STB levels in that environment.

Environment	Background	STB (Flag leaf direct infection)	STB (Flag leaf from base)	Height	SPAD	Leaf area	1000GW
Seedling tests	16 FLLO	-			L>F		
	24 FLLO	-			L>F		
	SPRI	-			R>S		
2012	16 FLLO			L>F	L>F		
	24 FLLO			L>F	L>F		
	SPRI						
2013 53	16 FLLO	-	-	F>L	L>F	L>F	
	24 FLLO	-	-	-	L>F	L>F	
	SPRI	-	-	-	R>S		
2014 Plastic glasshouse	16 FLLO	-	L>F	L>F	-	L>F	
	24 FLLO	-	L>F	L>F(#)	-	L>F	
	SPRI	-	R>S	R>S	R>S	R>S	
2014 Church farm field trial	16 FLLO		L>F	-	L>F	L>F	L>F
	24 FLLO		L>F	-	L>F	L>F	L>F
	SPRI		-	S>R	R>S	-	R>S
2014 Morley field trial	16 FLLO		L>F	L>F	L>F	-	L>F
	24 FLLO		L>F	-	L>F	L>F	L>F
	SPRI		-	S>R	R>S	-	R>S
2014 Ireland	16 FLLO		L>F				
	24 FLLO		L>F				
	SPRI		R>S				
2015 Plastic glasshouse	16 FLLO		F>L	F>L	F>L	-	
	24 FLLO		F>L	F>L (#)	F>L	-	
	SPRI		-	-	R>S	S>R	
2015 Morley field trial	16 FLLO		L>F	L>F	L>F	-	L>F
	24 FLLO		L>F	-	L>F	-	L>F
	SPRI		-	-	R>S	S>R	R>S

Table 7.2.1: Summary of main traits for comparison between STB and disease escape candidate traits. Data presented as alleles with significantly greater value indicated by the letter of the parent that provided that allele. (F = Flame allele, L = Longbow allele, S = Spark allele and R = Rialto allele). Data chosen for inclusion based on relevance to the discussion below. (#) used to indicate trends that whilst in-significant are of interest for the discussion. Tested traits that were not significant are marked with a dash –.

Leaf area was a promising candidate trait in 2014 as it showed the same pattern of results as the STB results. In the 2014 plastic glasshouse experiments the Longbow and Rialto alleles were associated with a greater leaf area and higher disease levels in Esc1. In the 2014 field trials the Longbow allele was associated with larger leaf area and increased STB in the upper canopy (S5), and there were no significant differences associated with 6A in either STB or leaf area in SPRI lines. However this pattern broke down in both backgrounds in 2015. In the FLLO NILs there was no significant difference in leaf area in either the field trial or the plastic glasshouse despite there being significant differences in flag leaf STB for both environments. There was also no significant difference in STB levels in the SPRI NILs despite the plants with the Spark allele having a significantly larger leaf area than Rialto.

The SPAD data showed similar patterns to the STB data for the Flame x Longbow NILs. The higher SPAD readings on Longbow were associated with higher levels of STB in all field experiments. Additional evidence for a relationship between these traits came from the 2015 plastic glasshouse where the effect of the 6A alleles on both the STB and leaf greenness reversed direction. However the SPAD data did not align neatly with the Spark x Rialto NILs data. There often were no significant differences in STB on the SPRI material despite the SPAD readings differing in the same environment. The SPAD readings operate in the same direction for both backgrounds, with Longbow and Rialto both having later emerging leaves that remained green later into the growing season.

This difference in SPAD readings could be caused by differences in senescence rate, leaf lifetime or leaf emergence date. Dark induced senescence tests showed no significant differences in senescence rate. Measurement of leaf lifetime and leaf emergence in seedlings showed no difference in leaf lifetime and significant differences in leaf emergence between the lines. Whilst leaf emergence itself wasn't measured in detail in all trials, the SPAD differences between lines seem to have reflected a difference in leaf emergence date rather than a changed senescence profile over the lifetime of the leaf.

Another factor to consider when analysing the data is the difference between the two Flame x Longbow families. The escape and field experiments both had a highly significant effect of family, corresponding to family 16 being more heavily infected with STB than 24. This difference was also found in the inoculated adult plant experiments indicating that the background of family 16 was more susceptible. There was also a strong effect of family on height, with plants in family 16 being significantly taller than those from 24. Taller plants had greater levels of disease escape than shorter plants, and thus the presence of higher disease levels in family 16 in experiments subject to disease escape means that there must be a counteracting effect that is stronger than the height difference. The max STB height data showed a greater degree of disease spread in the family 16 lines than in family 24, indicating that the resistance/susceptibility difference may have increased spore transfer by allowing more inoculum to build up in the lower canopy. The increase in disease spread in family 16 may also have been affected by the greater leaf size of family 16 relative to 24 in some environments. It is also worth noting that because the way the QTL was identified using STB scores adjusted for height, height cannot be the sole cause of the escape effect at the region, but may be affecting the differences between the families and backgrounds.

Considering the Flame x Longbow NILs by themselves, the data on leaf emergence is the trait that most closely fits the STB results. However the relationship between leaf emergence and the Spark x Rialto lines is less clear. In the majority of STB tests, there were no significant differences caused by the different 6A alleles in SPRI despite the presence of the differing emergence. However, when significant differences in SPRI did occur (Esc1 and Ireland) they followed the same trend as the FLLO results for both STB and leaf emergence.

7.3 Main Hypothesis

The relationship between the 6A alleles and STB levels appears to be that there are differences in disease levels when the plants are infected at the base but not when leaves are inoculated directly. This supports the hypothesis that the 6A region does not contain a novel resistance gene, but instead affects disease escape. From the measurement of several physiological and developmental traits on the near isogenic

lines, three hypotheses for this difference in disease escape appear. Hypothesis 1 is that differences in leaf area between the 6A lines, leads to differences in levels of STB. Hypothesis 2 is that higher levels of STB are caused by later emerging leaves of the plants with either the Longbow or Rialto allele. Hypothesis 3 is that an untested factor causes the escape difference between the lines. For example, a trait that we were unable to test was leaf waxiness. Leaves of varying waxiness could affect disease escape by changing the likelihood of splashed spores remaining on the leaf they land on. This effect would not be detected in the inoculated experiments due to the addition of Tween-20 to the inoculum yet would be present in the escape and field trials. This or any number of other traits could be the true cause of the STB differences, as there is no demonstration of causation for hypothesis 3.

Hypothesis 1 is that leaf area caused the differences in disease escape. This explanation for this is that the net rate of successful spore movements may be greater in plants with larger leaves because of the greater target area presented by larger leaves. In addition to this, more spores may be produced by a larger leaf that has the same percentage coverage of septoria. This means that fully infected leaves at the base of the canopy produce more spores and thus increase the potential for spores being transferred to new leaf material.

There are a few results that indicate that hypothesis 1 is not the sole cause of the differences in STB. In the 2015 field trial there were significant differences in leaf area between the Spark and Rialto alleles without an associated difference in STB levels. This can be explained in many ways due to the many environmental factors that could lead to the effect of area on STB not occurring at a noticeable level. Leaf area cannot be the sole escape trait responsible for the differences in STB levels, as otherwise there would not be a difference in levels of STB between the Flame and Longbow NILs in 2015, when there was no equivalent difference in area. In addition, leaf area was never significantly higher in lines with the Rialto allele. In the SPRI material, leaf area differences were always non-significant or larger with the Spark allele.

To assess the plausibility of hypothesis 2, previous work on the relationship between the timing of crop development and disease escape needs to be considered. It was

been suggested that the main effect of leaf emergence and heading date on disease levels occurs via altering the length of time the leaf has been infected prior to scoring (Van Beuningen and Kohli, 1990). Given that scoring of disease symptoms is typically done on a set timepoint, rather than relative to leaf age, later emerging leaf tissues would be predicted to have lower disease levels due to the reduced time for disease development after infection. This explanation for the effect of leaf emergence makes intuitive sense, because leaves that are infected with the same initial amount of inoculum and have less time for the disease to develop prior to scoring will have fewer symptoms on the scored leaf. This process places the emphasis on horizontal secondary infections and amount of multiplication within the same leaf/leaf layer. This is important as most damage to the leaf is usually caused by the second or third cycle of multiplication (Shaw and Royle, 1993). However it rests on the assumption that there are no differences of practical significance in vertical spread between the lines. This assumption would be correct in situations where there are viable opportunities for disease spread during the emergence of the relevant leaf layers. This is shown on the left side of Figure 7.2.1.

The length of infection hypothesis for the effect of heading date and leaf emergence on disease escape predicts the opposite relationship to the one found at the associated with the 6A alleles, where the later emerging leaves in the Longbow and Rialto lines had increased or equal disease symptoms rather than less. For hypothesis 2 to contribute to the difference in STB, the following relationship between leaf emergence and STB is proposed. Later leaf emergence reduces STB levels by limiting the time for secondary infection and horizontal transfer, however later leaf emergence also increases STB levels by increasing vertical transfer in conditions where heavy rainfall occurs later after leaf development. Thus the effect of leaf emergence on STB levels depends on the weather conditions around leaf emergence and other factors that influence the relative importance of vertical transfer and secondary infection.

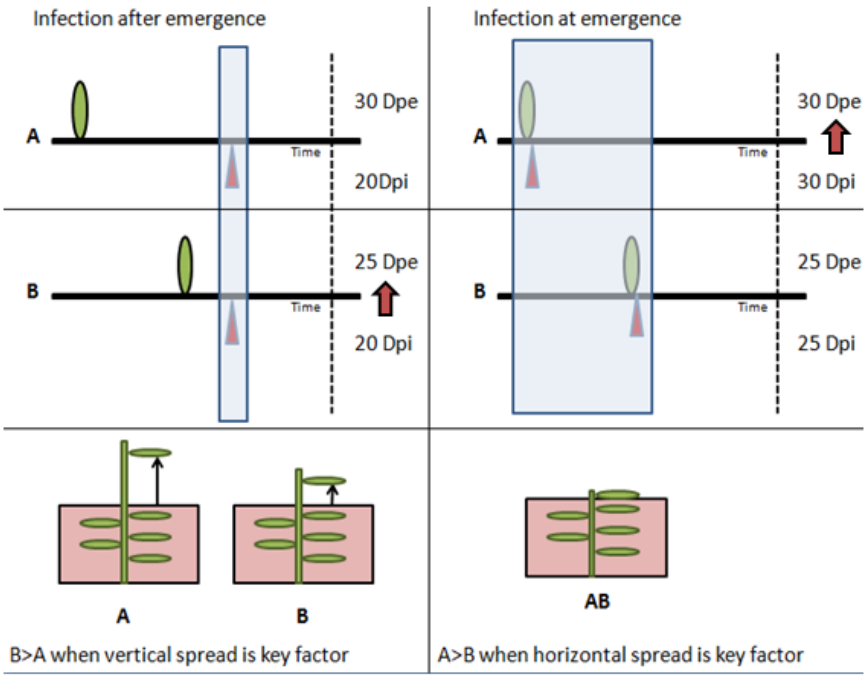

Figure 7.2.1: Potential interactions between leaf emergence and disease escape. Line A has earlier leaf emergence than B, as indicated by the leaves on the black timelines. STB spreading onto the newly emerged leaf is indicated by the red triangle, and the scoring of the plant by the dotted line. Heavy splashy rainfall is shown by the blue shaded box. Dpe stands for days post leaf emergence and Dpi for days post infection. The red arrow indicates which line ends up with higher levels of STB. In the bottom half of the diagram plants are shown at the point of STB spread. The black arrow represents the distance the STB has to travel to infect the new leaf layer. The pink box shows area of infected younger leaves. The left hand side of the diagram shows early emerging leaves getting less STB due to disease escape prior to an opportunity for spore transfer. The right hand side of the diagram shows early emerging leaves getting more STB due to a greater amount of time between infection and scoring.

This theory works on the assumption that vertical spore transfer does not occur continually and requires discrete episodes of heavy rainfall. Depending on the conditions the crop is growing in heavy rainfall can be a frequent or rare event. Comparing two lines A & B with different leaf emergence dates, if conditions are not suitable for spore transfer until both leaves have emerged, they will be at the same Dpi when scored (Figure 7.2.1). This will mean that both lines will have the same

opportunity for lesion growth and spread within that leaf layer. However, they are unlikely to have the same initial amount of infection under this scenario, as the younger leaf is likely to be lower in the canopy and thus closer to the existing infected material than older emerged leaves (Shaw and Royle, 1993). Increasing the distance between the emerging leaf material and existing lesions leads to less spore transfer to the emerging material (Lovell et al., 2004).

This explanation interacts with the relationship between leaf emergence timings and stem extension. It works on the principle of stem extension being shifted at the same rate as leaf emergence, so that the relative distance between emerging tissue and infected tissue is different between the lines. It is also worth noting that when the plant is fully mature, the leaves are at the same height, so if heavy rainfall does not happen until then, the differences will also not occur, leading to a set timeframe when this effect can occur. The effect also would not occur if heavy rainfall occurs around the emergence of the first leaf and a subsequent dry period prevents similar spore transfer occurring for the later emerging leaf (Shaw and Royle, 1993).

Shaw and Royle (1993) showed that secondary infection within the same leaf layer was the main determinant of the level of STB on the flag leaf, with only a few lesions from vertical transfer being required. Due to the inoculum spread decreasing exponentially with height (Shaw, 1991) serious infection of any given leaf layer is likely to occur when the leaf is emerging and is still close to other infected leaf material. This leads to the relationship between rainfall and leaf emergence dates being crucial to the effect of leaf emergence on the final level of disease. This is supported by the previously found relationship between STB and the number of rainy days in May and June (Polley and Thomas, 1991). The two scenarios shown in Figure 7.2.1 are this effect taken to the extreme. In practice both the effect of leaf emergence on vertical transfer and the effect on time for secondary multiplication will contribute to the final levels of STB. The relative importance of these two effects is likely to be determined by the relationship between leaf emergence date and spore transfer events. In Shaw & Royle (1993) the differences in secondary multiplication were dominant, but in the 6A material the effect of vertical transmission appears to be causing the greater effect.

This decreased length of time for disease development prior to scoring in leaves that emerge later is presumed to be behind the strong interaction shown between heading date and STB in the work of Van Beuningen and Kohli (1990). Experiments in Kenya, showed earlier developing lines having far greater STB than later developing lines due to this effect. Scoring by leaf developmental stage showed no significant differences, but infection occurred at the same developmental stage in each leaf, which is consistent with the model in Figure 7.2.1 as whilst there is no difference in Dpi, there was no difference in leaf stage of infection either (Arama et al., 1999). In other studies there were differences in the relationship between emergence dates and disease escape consistent with the theory of the two potential effects of leaf emergence altering in relative importance depending on external factors. In Simón et al (2005) field differences in STB were attributed to disease escape due to the lack of significant differences in inoculated experiments. However the direction of the association between STB and heading date varied between years and conditions. Heading date also had a minor and variable effect in Arraiano et al (2009). That weather conditions and other external factors shift the ratio between importance of primary and secondary infections and thus the effect of leaf emergence, is consistent with the established literature.

SPRI data and the hypothesis

The lack of significant differences caused by the 6A alleles in flag leaf STB for the Spark x Rialto background is surprising. The very clear difference found in the Irish field trial is consistent with them having an effect on STB in field conditions. One theory to explain this is that the greater resistance in the Spark x Rialto background means that the differences in disease escape cannot be seen unless under high disease pressure. Ireland is known for having greater levels of STB than the UK, leading to the difference being visible there but not in Norfolk. This theory is also supported by the Flame x Longbow effect also being more distinct under the higher disease pressure of the Irish trials. In addition the lower sowing rate in the Irish trial may have influenced the effect of leaf emergence on STB. The greater spacing between plants may have caused vertical transfer to become more important relative to horizontal transfer. This may

have affected the increased difference in STB between the 6A lines due to a shift in the relative importance of the two responses to changes in leaf emergence.

In England the lack of significant difference in STB in the SPRI material could be due to the two directions of the leaf emergence effect balancing due to how the timing of heavy rainfall occurred relative to the leaf emergence.

Plastic glasshouse data and the hypothesis

The design of the plastic glasshouse experiment has implications for relating its data to the hypotheses. The late infection of the base compared to the field trials meant that relatively little disease will develop prior to emergence of the upper leaves. This may have meant that the majority of spore transfer events occurred between leaves that were already established. This is supported by the relatively low disease scores on the flag leaves and the high frequency of leaves with no symptoms indicating that the symptoms seen come from infrequent vertical transfer with little opportunity for secondary infection. This may mean that any differences found in the plastic glasshouse were due to traits other than leaf emergence, such as leaf area and height.

The leaf area data fits with the pattern shown in the 2014 STB results in Esc1, indicating that it may be affecting disease escape in that experiment. The results from Esc 2 are harder to explain, as the trend in STB levels was in the opposite direction to all preceding experiments, with higher disease levels in Flame than Longbow. There was also a very large difference between the two FLLO families, with plants with family 16 getting much more disease. This cannot be explained by leaf area data as there was not a significant difference between the Flame and Longbow alleles for leaf area in the 2015 plastic glasshouse. Plant height cannot explain the result either, as the increased height associated with Family 16 and the Flame allele, were also associated with greater levels of STB in, the opposite of the known effect of height. The reason for the dramatically different results in the 2015 plastic glasshouse is unknown. It is possible that the plants were slightly stressed in the summer due to the timing of watering system being adjusted, for the weather, less frequently than the preceding year, but this seems unlikely to be the sole cause of such a dramatic shift.

Returning to the hypothesis

When trying to determine what physiological and developmental traits are causing the difference in disease escape in the 6A NILs, it is important to remember that the effect may be caused by a combination of traits. Of all the traits tested, the leaf emergence difference fits with the STB results the best. However, that does not mean that in the experiments where differences in leaf area did occur, that they could not have also been affecting disease escape in that particular experiment. The disease escape effect may result from a combination of differences in height, leaf area, leaf emergence and possibly other traits. The data collected in this thesis shows that disease escape differences were found associated with the 6A region and that of the tested traits that may be contributing to the escape, the one that most closely fits the data is the leaf emergence.

The proposed mechanism for how for the leaf emergence effect of the 6A locus affects STB levels is dependent on the relative influence of two different effects. Later leaf emergence could increase STB levels because there would be a smaller distance to sporulating lesions when transfer occurs, and also decrease STB levels by reducing the time for secondary infection. It is proposed that in our field experiments the vertical transmission effect was stronger in the Flame X Longbow material leading to the later emerging flag leaves getting more disease. Existing literature on the effect of leaf lifetime shows negative associations between heading date and STB, consistent with the theory that the change in time for secondary infection is usually the larger effect (Van Beuningen and Kohli, 1990, Camacho-Casas et al., 1995) . However some studies showed variation in the degree of association between STB and emergence dates, with the effect being absent or variable even in large studies with lines with varied heading dates, (Simón et al., 2005{Arraiano, 2009 #62, Arraiano, 2015)}. This data is consistent with variation in degree between contrasting effects.

7.4 Yield and STB

The yield data on 1000GW and grain width showed increased yield associated with the Rialto allele as previously found for Spark x Rialto NILs (Simmonds et al., 2014). In the FLLO 16 and FLLO 24 backgrounds there was increased grain width and 1000GW

associated with the Longbow allele. That the Longbow allele gave the same response as the Rialto allele adds support to the association between the 6A QTL and increased yield by confirming its presence in an alternative background.

SPRI recombinant lines were also tested in our experiments. Whilst not replicated very heavily, the 1000GW data had increased grain weight between SR-12 and SR-17 (Figure 4.3.12).This pattern in the recombinants supports the gene for yield being found between PSP3071 and BS00003581. There was no corresponding pattern in the recombinants for the STB data. This isn't necessarily due to the traits being separate, as the experiments the recombinants were tested in didn't show significant differences between the Spark and Rialto alleles. The SPADmeter data and leaf area data for the SPRI recombinants in the three experiments also did not show the same pattern as the recombinants did for the yield data. This does not prove that neither of these traits is pleiotropic with the yield increase, but if they had shown the same pattern as the recombinants it would have narrowed them down to a similar region. An indication that there is a STB effect that occurs further along on the 6A chromosome comes from the recombinant data on levels of STB. Whilst not significantly differing between the Spark and Rialto alleles of 6A, STB levels were significantly increased in the SR-6 and SR-21 recombinants (Figures 5.3.3, and 6.3.7). No explanation for the increase in STB associated with these lines is currently available; however the absence of the effect in the lower canopy supports it being based on disease escape. The STB effect in these recombinants cannot be the cause of the STB differences associated with 6A in the Flame x Longbow NILs, as it occurs outside of region tested in the FLLO NILs, which are narrower than the SPRI NILs.

The initial premise of this project was to investigate the potential trade-off between the yield effect and the STB effect at the locus. The trade-off was expected to occur due to the high yielding Longbow and Rialto alleles at the 6A locus also being associated with higher levels of STB. The usefulness of the 6A QTL's effect on increasing thousand grain weight would be dependent on the extent to which the increase in levels of STB reduces yield. In the field trial experiments, the Longbow and Rialto alleles produced larger grain in both the treated and untreated trial. The effect of the 6A locus on STB levels was sufficiently small that there was not a significant

interaction between the effect of the allele on yield and the type of trial being performed (Table 4.3.11). The lack of a yield penalty associated with the Longbow and Rialto alleles in the untreated trials supports the use of the 6A QTL to increase 1000 grain weight even if the traits are pleiotropic. However, whilst the trade off at the locus was not important in our field trials, the costs and benefits of this locus are likely to be highly dependent on the environment and the trade-off may be larger under higher disease pressure, tipping the balance of the trade-off in the other direction.

7.5 Environmental factors

The escape experiments in the plastic glasshouse had lower levels of STB than either the directly inoculated plants or the field trials. The later infection date than in the field trials have left a smaller amount of time to build up inoculum levels through cycles of re-infection to occur and made transfer more difficult due to less time between infection and leaf emergence. However an additional factor that differs between the plastic glasshouse and the field trial is the presence of wind. In field conditions heavy rainfall is frequently accompanied by wind which moves the leaves of the plant increasing the contact between neighbouring leaves. Whilst heavy rainfall was simulated by frequent overhead watering in the plastic glasshouse, the walls of the tunnel and the barriers between plots meant that there was almost no movement due to wind. Lovell (2004) showed that even in the absence of heavy watering, the presence of mist and wind was sufficient to encourage contact spread of STB. Whilst the escape tests showed that wind is not essential for rain based spore transfer the low scores in the windless environment indicate its importance in allowing disease spread to occur.

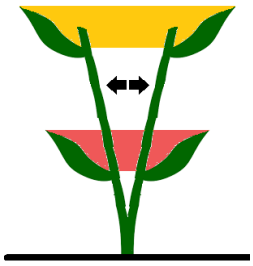

Figure 7.5.1: Model of effective leaf space including plant movement. The red marked area and orange marked area show the planes of potential spore interception of the second and flag leaf respectively.

In addition to windy conditions increasing the likelihood of direct contact between leaves, the relative position of the leaves changes under windy conditions, due to the pivoting of the plant from the stem base (Figure 7.5.1). This could increase spore transfer to the upper leaves by lowering the distance between the leaves and sources of infection.

7.6 Returning to resistance

The hypothesis detailed above in the discussion is proposing a potential disease escape based explanation for the STB data based on the physiological and developmental readings recorded for the same experiments. However there is an alternative explanation for the disease results. Instead of the disease escape mechanism that has a strong genotype by environment component, it could be a resistance effect that is also dependent on the environment. If this was the case, the resistance effect would have to only occur in adult leaves in field conditions, as chapter 3 showed no resistance differences in seedlings or the glasshouse. Additionally the GxE effect would have to account for the SPRI difference occurring in Ireland but not in Norfolk. The favouring of the disease escape explanation over this prospective variable resistance comes from two pieces of evidence. Adult onset resistance would be expected to affect all adult plant leaves equally, whereas the field trials showed significant effects only in the highest leaves. Adult leaves lower in the canopy (such as in S3) did not show significant differences. Additionally, a resistance difference would predict that the basally inoculated glasshouse plants would behave similarly to the flag leaf inoculated plants in the glasshouse. Thus the different levels of flag leaf STB in plants inoculated in lower leaves in the glasshouse indicate the alternative explanation (Chapter 6).

7.7 Implications for future work

Work on the 6A locus

The results from this project have led to the a hypothesis that the 6A region may be affecting STB due to the interaction between leaf emergence, timing of rainfall and the height of leaf at infection. This is inferred based on the patterns shown in the data and not demonstrated directly. A focused study on the 6A lines in the field taking readings of daily rainfall, precise leaf emergence dates and leaf spacing in late spring could be used to test the hypothesis. Further confirmation of the effect could also be achieved by running inoculated field trials alongside the naturally infected plots.

In field conditions, leaves of the near 6A NILs showed differences in colouration not noticeable in glasshouse and plastic glasshouse conditions. This may be due to different wax profiles of the leaves. Testing the composition of the leaf surface in the 6A lines would allow investigation of the hypothesis that the differences in waxiness between the lines affects disease escape by altering spore and droplet adhesion.

It is also worth mentioning that the interaction between the 6A alleles and disease escape is not necessarily specific to STB and may occur with any splash borne disease. Growing these NILs in an environment where *Stagnospora nodorum* is the major pathogen to test if disease differences occur in the upper leaves would demonstrate if it could be applied more broadly.

The yield effect found associated with psp3071 on chromosome 6A is currently being studied by Dr Cristobal Uauy's group at the John Innes Centre, including further mapping of the gene and investigation of its mechanism.

Disease escape

In addition to further work on the 6A material, these experiments raise questions about what other physiological and developmental traits may also affect disease escape. There may be a lot of potential for reducing levels of disease by growing plants with ideotypes that reduce disease escape. The work in this project was performed by looking at a locus that affects disease levels and trying to identify a trait that may be

causing it. The opposite procedure would be very useful in looking for traits that cause differences in disease escape. Breeding near isogenic lines that differ for a trait of interest then testing for their effect on disease escape by comparing the difference between direct inoculation and natural infection. This could be performed on traits that were not shown to be significantly different in the 6A material but plausibly could affect disease escape such as leaf angle. If appropriate genetic material was available, performing tests on lines that had reliable differences in leaf area, leaf lifetime and leaf waxiness would aid knowledge in finding ideotypes that increase disease escape.

The advantage of investigating traits to see if they affect disease escape is that unlike identifying novel resistance genes, finding new sources of disease escape cannot lead to an arms race with the pathogen. If leaves that are shorter and wider with a more prostrate position lead to a reduction in spore movement, the pathogen will not be able to induce the rainfall to splash differently on these types of leaf. Differences in disease level from increased disease escape may impact yield, due the alteration of the physiological traits. However their durability may make then a worthwhile consideration for future plant breeding efforts.

8 Appendices

Line	PSP 3071	BS000 022947	BS000 22992	BS000 03881	BS000 04377	BS000 01132	BS000 03581	BS000 09783	BS000 09871	BS000 09988	BS000 23089
	Initial	M5	M2	13	12	14	18	17	15	16	M3
16A1*	F	L	L	L	L	F	F	F	F	F	F
16A2*	F	L	L	L	L	F	F	F	F	F	F
16A3*	F	L	L	L	L	F	F	F	F	F	F
16A4*	L	L	L	L	L	L	L	L	L	L	L
16A5*	F	L	L	L	L	F	F	F	F	F	F
16A6	H	L	L	L	L	H	H	H	H	H	H
16B1	H	L	L	L	L	H	H	H	H	H	H
16B2	H	L	L	L	L	H	H	H	H	H	H
16B3*	L	L	L	L	L	L	L	L	L	L	L
16B4*	L	L	L	L	L	L	L	L	L	L	L
16B5*	F	L	L	L	L	F	F	F	F	F	F
16B6	H	L	L	L	L	H	X	H	H	L	H
16B7	H	L	L	L	L	H	X	H	H	L	H
16B8*	L	L	L	L	L	L	L	L	L	L	L
16B9*	L	L	L	L	L	H	X	X	L	L	L
16B10*	F	L	L	L	L	F	F	F	X	X	F
16B11	H	L	L	L	L	L	X	H	H	H	H
16B12	H	L	L	L	L	X	X	H	H	H	H
16B13*	L	L	L	L	L	L	L	L	L	L	L
16B14	H	L	L	L	L	H	H	H	H	H	H
16B15	H	L	L	L	L	H	H	H	H	H	H
16B16*	L	L	L	L	L	L	L	X	L	X	L
16B17	H	L	L	L	L	H	H	H	H	H	H
16B18	H	L	L	L	L	H	X	H	H	X	H
24A1	L	L	L	L	L	L	L	L	L	L	L
24A2	L	L	L	L	L	L	L	L	L	L	L
24A3	L	L	L	L	L	L	L	L	L	L	L
24A4	L	L	L	L	L	L	L	L	L	L	L
24A5	L	L	L	L	L	L	L	L	L	L	L
24A6	L	L	L	L	L	L	L	L	L	L	L
24A7	L	L	L	L	L	L	L	L	L	L	L
24A8	L	L	L	L	L	L	L	L	L	L	L
24A9	L	L	L	L	L	L	L	L	L	L	L

Table 8.1.A Genotyping of Env54 2012 with early Kaspar data.* = The lines used in the subsequent multiplication trial. L and F refer to the maker showing the same SNP as the Longbow or Flame parent respectively. H represents a heterozygote, and X an unclear or unreliable result.

Line	PSP 3071	BS00 022947	BS000 22992	BS000 03881	BS000 04377	BS000 01132	BS000 03581	BS000 09783	BS000 09871	BS000 09988	BS000 23089
24A10	L	L	L	L	L	L	L	L	L	L	L
24A11	H	L	L	L	L	H	H	H	H	H	H
24A12	L	L	L	L	L	L	L	L	L	L	L
24A13	L	L	L	L	L	L	L	L	L	L	L
24A14	L	L	L	L	L	L	L	L	L	L	L
24A15	L	L	L	L	L	L	L	L	L	L	L
24A16	L	L	L	L	L	L	L	L	L	L	L
24A17	L	L	L	L	L	L	L	L	L	L	L
24B1*	L	L	L	L	L	L	L	L	L	L	L
24B2*	L	L	L	L	L	L	L	L	L	L	L
24B3	H	L	L	L	L	L	H	H	H	H	H
24B4	H	L	L	L	L	L	H	H	H	H	H
24B5*	F	L	L	L	L	F	F	F	F	F	F
24B6	H	L	L	L	L	X	H	H	H	H	H
24B7*	L	L	L	L	L	L	L	L	L	L	L
24B8*	L	L	L	L	L	L	L	L	L	L	L
24B9	H	L	L	L	L	H	H	H	H	H	H
24B10	H	L	L	L	L	H	H	H	H	H	H
24B11*	F	L	L	L	L	F	F	F	F	F	F
24B12*	L	L	L	L	L	L	L	L	L	L	L
24B13	H	L	L	L	L	H	H	H	H	H	H
24B14	H	L	L	L	L	H	H	H	H	H	H
24B15*	L	L	L	L	L	L	L	L	L	L	L
24B16*	L	L	L	L	L	L	L	L	L	L	L
24B17*	F	L	L	L	L	F	F	F	F	F	F
24B18*	L	L	L	L	L	L	L	L	L	L	L
24C1*	F	L	L	L	L	F	F	F	F	F	F
24C2*	L	L	L	L	L	L	L	L	L	L	L
24C3*	F	L	L	L	L	F	F	F	F	F	F
24C4*	L	L	L	L	L	H	L	H	L	L	L
24C5	H	L	L	L	L	X	H	H	H	H	H
24C6	H	L	L	L	L	X	H	H	H	H	H
24C7*	L	L	L	L	L	L	L	L	L	L	L
24C8	H	L	L	L	L	H	H	H	H	H	H
24C9	H	L	L	L	L	H	H	H	H	H	H

Table 8.1 Genotyping of Env54 2012 with early Kaspar data. * = The lines used in the subsequent multiplication trial. L and F refer to the maker showing the same SNP as the Longbow or Flame parent respectively. H represents a heterozygote, and X an unclear or unreliable result.

Line	PSP 3071	BS00 022947	BS000 22992	BS000 03881	BS000 04377	BS000 01132	BS000 03581	BS000 09783	BS000 09871	BS000 09988	BS000 23089
24C10	H	L	L	L	L	H	H	H	H	H	H
24C11	H	L	L	L	L	H	H	H	H	H	H
24C12	H	L	L	L	L	H	H	H	H	H	H
24C13*	L	L	L	L	L	L	L	L	L	L	L
24C14	H	L	L	L	L	H	H	H	H	H	H
24C15*	L	L	L	L	L	L	L	L	L	L	L
24C16*	F	L	L	L	L	F	F	F	F	F	F
24C17	H	L	L	L	L	X	H	H	H	H	H
24C18	H	L	L	L	L	X	H	H	H	H	H
24D1*	L	L	L	L	L	L	L	L	L	L	L
24D2*	F	L	L	L	L	F	F	F	F	F	F
24D3*	L	L	L	L	L	L	L	L	L	L	L
24D4	H	L	L	L	L	X	L	H	H	L	H
24D5*	F	L	L	L	L	F	F	F	F	F	F
24D6	L	L	L	L	L	X	L	L	H	L	H
24D7	L	L	L	L	L	L	L	L	L	L	X
24D8*	F	L	L	L	L	F	F	F	F	F	F
24D9	F	L	L	L	L	F	F	F	F	F	F
24D10	L	L	L	L	L	X	X	L	L	L	X
24D11	L	L	L	L	L	L	L	L	L	L	L
24D12	H	L	L	L	L	H	H	H	H	H	H
24D13	L	L	L	L	L	X	L	L	L	L	L
24D14*	F	L	L	L	L	F	F	F	F	F	F
24D15	H	L	L	L	L	H	H	H	H	H	H
24D16*	F	L	L	L	L	F	F	F	F	F	F
24D17*	F	L	L	L	L	F	F	F	F	F	F
24D18	H	L	L	L	L	X	X	H	H	X	X

Table 8.1 Genotyping of Env54 2012 with early Kaspar data. * = The lines used in the subsequent multiplication trial. L and F refer to the maker showing the same SNP as the Longbow or Flame parent respectively. H represents a heterozygote, and X an unclear or unreliable result.

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