# Effects of temperature on wheat-pathogen interactions

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## Abstract

Climate change is affecting UK agriculture, and research is needed to prepare crops for the future. Wheat is the UK's most important crop, and needs to be protected from losses caused by disease.

While direct effect of the environment on pathogen spread is often reported, effect of the environment on host defence is not. Many wheat resistance genes are temperature sensitive and these were used as a starting point to investigate defence temperature sensitivity in wheat starting with yellow rust resistance gene *Yr36*, previously shown to be temperature-sensitive. The effect of temperature on resistance was shown to be independent of *Yr36* in breeding line UC1041, and was more likely to be due to a previously-uncharacterised background temperature sensitivity. These results suggest that temperature changes, rather than thresholds, might influence some disease resistance mechanisms. Understanding this phenomenon could enable the breeding of more stable defence in crops.

In order to gain further insight into how temperature changes influence resistance, plants were grown under different thermoperiods and challenged with different types of pathogens; Results showed that resistance to multiple pathogens in one cultivar Claire was enhanced under variable temperatures, compared to constant temperatures. Taken together, the research presented revealed that defence temperature sensitivity in plants is much more complex than previously thought, considering that both temperature changes and different thermoperiods can influence aspects of wheat defence.

To ascertain which research approaches will be most valuable in preparing for climate change, the effect of the environment on take-all was also investigated. Vulnerable periods for wheat from the threat of take-all development were identified by analysing historical datasets, and controlled environment experiments. Results showed a relationship between initial post-sowing temperatures and spring take-all levels in 2$^{nd}$ 3$^{rd}$ or 4th winter wheats, depending on the location.

The work on yellow rust resistance and take-all both identify vulnerable periods for wheat caused by the environment, be it weakening of host defence responses, or increased threat from disease pressure. Further characterisation and understanding of vulnerable periods will be essential to control disease outbreaks under an increasingly unstable climate.

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# Contents

## Abbreviations

| | |
|---|---|
| APR | adult plant resistance |
| *Bgt* | *Blumeria graminis* f. sp. *tritici* |
| BLAST | Basic Local Alignment Search Tool |
| cDNA | complementary DNA |
| CE | controlled environment |
| CEBiP | chitin elicitor binding protein |
| CER | controlled environment room |
| CFU | colony forming units |
| $C_t$ | threshold cycle |
| DNA | deoxyribonucleic acid |
| dpi | days post inoculation |
| ETI | effector-triggered immunity |
| f. sp. | formae specialis |
| FLS2 | flagellin-sensitive-2 |
| hpi | hours post inoculation |
| HR | hypersensitive response |
| HSP | heat shock protein |
| HTAP | high temperature adult plant (resistance) |
| JA | jasmonic acid |
| L | litre |
| Log | logarithm |
| Lr | leaf rust |
| NBS-LRR | nucleotide-binding site leucine-rich repeat |
| MAMP | microbe-associated molecular pattern |
| Mol | molar |
| mRNA | messenger RNA |
| NCBI | National Centre for Biotechnology Information |

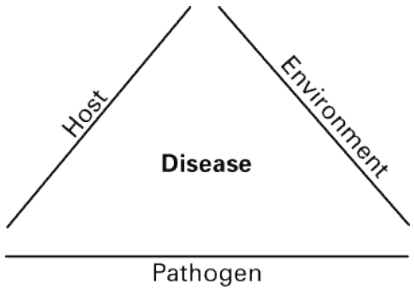
| | |
|---|---|
| NIL | near-isogenic line |
| OD | optical density |
| PAMP | pathogen-associated molecular pattern |
| PCR | polymerase chain reaction |
| PR | pathogenesis related |
| PRR | pattern recognition receptor |
| *Pst* | *Puccinia. striiformis* f. sp *tritici* |
| PTI | PAMP-triggered immunity |
| pv | pathovar |
| QDR | quantitative disease resistance |
| qRT-PCR | quantitative reverse transcription |
| R gene | resistance gene |
| RH | relative humidity |
| RNA | ribonucleic acid |
| SA | salicylic acid |
| SD | standard deviation |
| SE | standard error |
| SSV | sub-stomatal vesicle |
| UKCP | *UK climate projections* |
| Yr | yellow rust |

# Chapter 1: General introduction

## 1.1 The disease triangle

Wheat is the UK's most widely grown crop, covering about 2 million hectares and producing around 15 million tonnes each year (DEFRA, 2009). Wheat yield is continuously reduced by damage from various diseases so it is essential to control losses by understanding occurrence of disease outbreaks. For a disease to occur, a combination of a susceptible host, a pathogen able to infect the host and a suitable environment are required. The disease triangle (Figure 1.1) simplifies this complex relationship (Stevens, 1960) and illustrates the delicate balance between the variables that are required for disease occurrence. Perhaps the least stable of the three variables, and most likely to influence the balance, is the effect of the environment on disease outbreaks. Environment; already constantly changing and impossible to control, is going to become even less stable with climate change. To aid with future wheat breeding strategies we need to know how the relationship between host, pathogen and the environment is affected both now and in the future.

**Figure 1.1 The disease triangle (Stevens, 1960)**




Current wheat disease management strategies vary depending upon which pathogen needs to be controlled. Breeding for resistant cultivars is a long-practised method for dealing with disease out-breaks but this alone is not sufficient to eliminate risk. Fungicides delivered as spray for foliar diseases such as yellow rust, *Septoria* or powdery mildew or seed treatment

for seed-borne diseases are used, usually in combination with resistant cultivars, to prevent spread of disease. Disease forecasting is also important for timely application of fungicides (Hardwick, 2006). Crop rotation is another common practise to prevent build-up of soil pathogens for diseases such as eyespot or take-all. These practices are constantly adapting with time, due to development of new technology, social-economic factors and changes in local climate. It is essential that we understand how the environment affects diseases, to manage wheat agriculture in a changing climate.

## 1.2 Plant disease and climate change

Climate is changing and this will affect plant diseases (Chakraborty and Pangga, 2004; Coakley *et al*., 1999; Garrett *et al*., 2006; Scherm and Coakley, 2003). It is unknown how diseases will be affected, but it is likely that it will be detrimental to some pathogens and beneficial to others, depending on their ability to adapt or re-locate (Garret *et al*., 2009). The UK is likely to face the emergence of diseases that are currently not a major threat to the wheat industry. For example brown rust or leaf rust caused by *Puccinia triticina* is present in the UK but is not generally a threat. The disease develops most successfully when free moisture is available and temperatures are around 20°C (Prescott *et al*., 1986). Recent reports suggest that warming UK temperatures are encouraging new isolates of brown rust and increased disease outbreaks (Farmers Weekly, 2007; Allison, 2011). A more distant threat is stem rust disease, caused by *Puccinia graminis*, mostly absent from the UK at present and typically found in warmer climates (Davies *et al*., 2007). In contrast, levels of *Stagonospora nodorum* which causes wheat leaf and glume blotch have declined in the UK in recent years (Eyal, 1999; BASF Cereal Pests and Diseases) and consequently Septoria leaf blotch caused by *Mycosphaerella graminicola* has replaced *S. nodorum* in importance. Although there are many factors that influenced this shift from *S. nodorum* to *M. graminicola* (such as a shift from deployment of varieties that were more susceptible to *S. nodorum* and less susceptible to *M. graminicola,* to those with the opposite pattern of responses (Arraiano *et al*., 2009)) there is evidence that environmental factors were key (BASF, The Encyclopedia of cereal diseases; Bearchell *et al*., 2005; Shaw *et al*., 2008). Although caution needs to be exercised on whether environment changes are key to changes in pathogen equilibrium, it is likely that this will be a result of climate change.

Pathogen population changes will affect agricultural management, so it is important to identify potential threats (Juroszek and von Tiedemann, 2013).

Harvell *et al* (2002) argue three hypotheses why pathogens will be influenced by climate change. They suggest that that rising temperatures will (i) increase pathogen development transmission, and generation number; (ii) increase overwinter survival and reduce growth restrictions during this period and (iii) alter host susceptibility. Indeed, there is plenty of evidence to suggest that environment can affect host defence directly (Huang *et al*., 2006; Plazek *et al*., 2001; Webb *et al*., 2010) but climate change will also influence crop physiology and canopy architecture (Pangga *et al*., 2013; Porter and Gawith, 1999; Savicka and Škute, 2010) which may indirectly affect plant disease levels by interfering with the pathogens' ability to infect host tissue or by creating a different type of microclimate that influences pathogen spread. Therefore consideration of host adaptation to environmental change is equally valuable in predicting future disease.

UK Government risk assessments recognise that impact on plant disease is an important factor in how climate change will affect agriculture as a whole and that it should be incorporated into crop risk models. The risk assessment indicates that the evidence for impact is weak, but acknowledge that this is largely due to disease treatment methods and improved agronomy masking the effect of climatic factors (Knox *et al*., 2012). Essentially, researchers and breeders will need to work together to assess impact and generate solutions. A common way to study the effect of environment on plant disease is by using controlled environment facilities to manipulate environmental variables to see how disease is affected (Brennan *et al*., 2005; Johannessen *et al*., 2005; Monteiro *et al*., 2009). This approach limits the investigation to the effect of one environmental variable only, but is necessary to isolate impact of that variable alone. Another approach is to manipulate environmental variables in the field (Latva-Karjanmaa *et al*., 2003; Roy *et al*., 2004). These studies may be more informative but are difficult to control, and costly. Numerous studies have tried to predict outcomes by generation of models using a combination of historic disease records and current or future climate predictions (Butterworth *et al*., 2009; Evans *et al*., 2008; Hannukkala *et al*., 2007; Madgwick *et al*., 2011). It is well-recognised that the absence of long-term disease data sets makes this approach challenging (Jeger and Pautasso, 2008). These models are helpful to agriculture provided pathogens do not evolve to tolerate new conditions, as increasing evidence suggests (Mboup *et al*., 2012; Milus *et al*

2009; Milus *et al* 2006; Peduto *et al* 2013). What's more, these approaches are generally concerned with the effect of environment on pathogen fitness and rarely consider the effect of environment on the crops' ability to fight the disease.

Several reports about the impact of climate change on specific diseases of wheat have been published in the last decade (Chancellor and Kubiriba, 2006; West *et al*., 2012). Most reports are undecided about future prevalence of specific diseases so it is important to continue to identify threats through modelling, so management strategies can be put into place. These strategies could be in the form of agricultural practices, fungicide development, or breeding of functional resistance in warmer, more variable climates. Breeding of new wheat cultivars currently takes between 10-25 years so it is essential that new approaches are discovered and implemented without delay.

## 1.3 Climate change in the UK

UKCP09 is the working name for *UK climate projections* (UKCP09, 2009) which is funded by the Department for Environment, Food and Rural Affairs (DEFRA). The public can access these projections freely through a website which can be used to produce customized data for a wide range of climate variables. UKCP09 is the fifth generation of climate information for the UK, and is the most recent and most comprehensive package available to date. The UKCP09 interface indicates that as the decade progresses, all of the areas in the UK are going to warm, more so in summer than in winter. Increases in mean temperature will be greatest in Southern parts of England and least in the Scottish Islands. The biggest change in precipitation during winter months is a decrease seen along the west side of the UK, while the most significant summer precipitation change is a decrease in the most southern parts of England (Murphy *et al*, 2009). Climate change is expected to increase average temperatures but an increase in the frequency of temperature extremes is also predicted (Semenov *et al*., 2007). UKCP09 predicts that by 2020, average annual temperature will increase by 1-3 °C and by 2080 it will have risen by up to 6 °C in some areas (Figure 1.2). Therefore heat and drought stress are the two main threats from both rising temperatures and reductions in precipitation frequency. While it is important to prepare wheat crops for both stresses, there is some evidence to suggest that heat stress, not drought, will be the

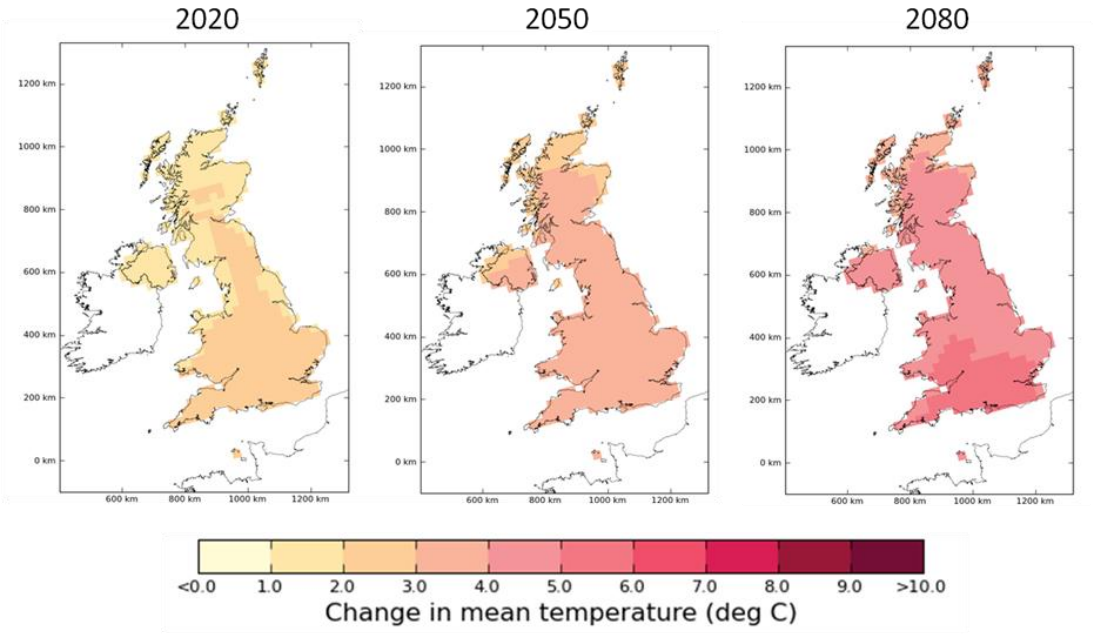



**Figure 1.2 UKCP09 temperature change prediction for the UK.** Images generated by UKCP09 show predicted change in mean temperature (°C) over land during the 21st century calculated from the baseline period of 1961-90. The images were generated using a medium emissions scenario at a 90% probability level.

main concern of climate change impact upon wheat in Europe and the UK (Semenov *et al*., 2008; Semenov and Shewry, 2011). In addition, due to predictions that precipitation changes will mostly be on the west side of the UK it may not be a key factor in preparing for impact of climate change on wheat diseases, considering the majority of UK wheat is grown in the East of England.

Crops are constantly exposed to temperature changes in the field on a yearly, daily and hourly basis. Although day and night temperatures are generally different, the daily and hourly patterns can also be extremely variable. For example, whereas sometimes there may be very stable periods with similar day and night temperatures occurring over multiple days, other periods are more sporadic with many temperature fluctuations occurring throughout the period (Figure 1.3). Where research has been conducted on how variation in temperature affects wheat crops, the general consensus is that UK yields are predicted to decrease from the onset of increased temperature variation (Mearns *et al*., 1997; Moot *et al*., 1996; Semenov and Porter, 1995). Models constructed by Semenov and Porter (1995) predicted that changes in climate variability would have a more profound effect on wheat yields than changes in mean temperature. To date, no research that has been conducted into how pathogens or plant defence respond to variations in temperature, as opposed to constant temperature.

## 1.4 Effect of environment on plant defence

Environmental impact on plant defence is difficult to determine in the field due to complicating factors such as multiple climatic variables, varying disease levels and perhaps most importantly pathogen-environment interactions. These factors make it extremely difficult to tease apart which environmental variables are important in maintaining functional crop defence. Temperature is the most recognised environmental factor that is able to affect resistance, but other environmental variables have been implicated. For example, elevated $CO_2$ can an affect resistance to *Colletotrichum gloeosporioides* in *Stylosanthes scabra* (Pangga *et al*., 2004), whilst leaf wetness duration has been shown to affect Rlm6-mediated resistance to *Leptosphaeria maculans* in *Brassica napus* (Huang *et al*., 2006). Carson and Vandyke (1994) demonstrated that light levels influenced defence in *Zea*

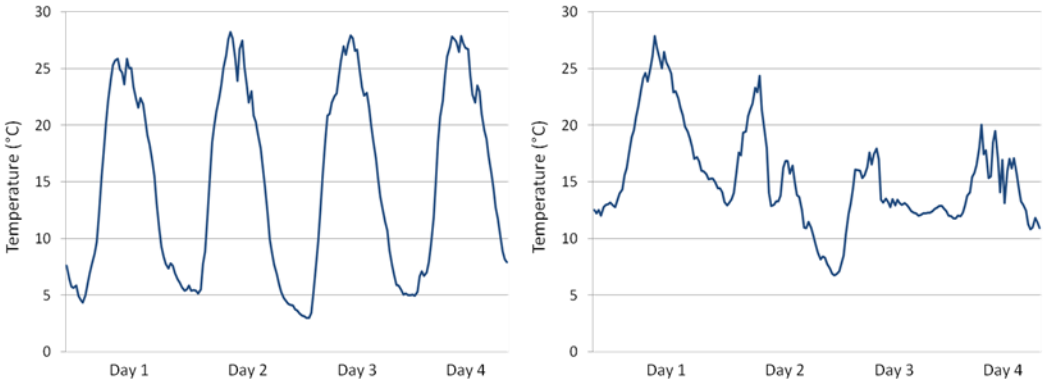



**Figure 1.3 Temperature readings taken from field plot monitoring.** Data show temperature readings collected from John Innes centre field trial site in 2011. Recordings were collected every 30 minutes using data loggers (Signatrol Ltd) and both show recordings from four consecutive days.

*mays* to *Exserohilum-turcicum* and the effect of ozone levels on resistance to a variety of fungal pathogens on various cereals has also been implicated (Plazek *et al*., 2001).

Plants have several layers of defence that provide protection against invading microbes. Pre-formed physical structures, for example cuticle thickness or leaf surface structures, can affect pathogen entry, and reduced access to nutrients can prevent proliferation. Plants can also recognise conserved pathogen (or microbe)-associated molecular patterns (PAMPs/MAMPs) by pattern recognition receptors (PRRs) which results in PAMP-triggered immunity (PTI; Jones and Dangl 2006). PTI results in induced defences including cell wall reinforcement, production of antimicrobial compounds and stomatal closure, which are sufficient to repel or deter most invading microbes (Melotto *et al*., 2006; Schwessinger and Ronald, 2012; Zipfel, 2009). Virulent pathogens have evolved to suppress PTI with effectors that interfere with PRR function or downstream signalling components. This first layer of defence is known as basal defence. Another level of defence is provided by R proteins which detect these effectors in effector-triggered immunity (ETI), a type of resistance typified by hypersensitive response (HR) and cell death (Jones & Dangl, 2006). This race-specific R-gene mediated resistance is complete but often short-lived due to mutation or loss of the specific effector from the pathogen (de Vallavieille-Pope *et al*., 2012; El Jarroudi *et al*., 2011).

In contrast, quantitative disease resistance (QDR) or partial resistance does not convey complete resistance, but is considered more durable due to reduced pressure on the pathogen to overcome defence mechanisms (Kou and Wang, 2010). QDR resistance is associated with phenotypes such as a delayed latent period or reduced pustule size (Herrera-Foessel *et al*., 2012; Rubiales and Niks, 1995). Most R genes are predicted to encode proteins with nucleotide-binding site and leucine-rich repeat (NBS–LRR) domains for effector recognition. However cloning of wheat QDR genes *Yr36* and *Lr34* have revealed that these genes do not fall under the major NBR-LRR class of genes suggesting that QDR mechanisms may be fundamentally different from those of R genes (Fu *et al*., 2009; Krattinger *et al*., 2009). QDR resistance may be conferred by a single or several genes (Ballini *et al*., 2008; Fu *et al*., 2009; Poland *et al*., 2009), and although currently not proved, some mechanisms of QDR conferred resistance may be provided by both the pre-formed and inducible PTI components of plant defence (Lloyd *et al*., in press). The influence

of temperature on the activity of R proteins, QDR genes and, more recently, proteins involved in basal defence has been demonstrated (Fu *et al*., 2009; Huang *et al*., 2006; Krattinger *et al*., 2009; Upchurch and Ramirez, 2011; Wang *et al*., 2009; Zhu *et al*., 2010).

R gene temperature sensitivity has been largely studied in model plant organisms. For example, the N gene of tobacco confers resistance to tobacco mosaic virus at 22°C but not at 30°C (Whitham *et al*., 1996). Likewise, the hypersensitive response conferred by Arabidopsis *RPW8* gene against powdery mildew is suppressed at temperatures above 30°C (Xiao *et al.,* 2003). Zhu *et al* (2010) identified NB-LRR receptor; suppressor of npr1-1 constitutive 1 (SNC1) as a temperature sensor in modulation of Arabidopsis resistance and growth; considering *snc1-1* mutants displays a constitutive defence response and growth defects at 22°C but not at 28°C (Yang *et al* 2004; Wang *et al.,* 2009). The authors hypothesise that temperature sensitivity in plants is largely regulated by NB-LRR coding genes rather than other signalling components due to a similar mutation in the N gene of tobacco, resulting in the lose loss of temperature sensitivity (Zhu *et al*.,2010). However there is evidence to suggest that the concept is more ambiguous due to evidence that other alleles and loci are able to modulate temperature sensitivity (Negeri *et al*., 2013). There are many examples of mutants that convey both growth and defence phenotypes that are temperature sensitive (Hua *et al*., 2001; Ichimura *et al*., 2006; Shirano *et al*., 2002). Indeed, defence and growth are intrinsically linked in nature due to defence being costly by taking resources away from growth and reproduction (Brown, 2002; van Hulten *et al* 2006; Walters *et al*., 2008). A recent review by Alcázar and Parker (2011) proposes that temperature sensitivity of defence in plants may have evolved to enable a plant to adapt to its local environment by balancing resources between growth and defence. They point out that, in contrast, 'priming' of resistance whereby previous exposure of plants to stress enables a faster response to subsequent stresses appears to be less costly (Traw *et al.,* 2007). Since there is evidence that priming can enhance a plant's response to both biotic and abiotic stresses, it is another example of how the environment can influence plant resistance upstream of specific NB-LRR receptors (Beckers and Conrath, 2007; Conrath *et al*., 2006).

There are many additional examples of R genes that are inhibited by high temperature in Arabidopsis but it is difficult to find examples of lower temperatures inhibiting R gene

mediated or any other aspects of defence. In cereals however, there is evidence to suggest that low temperatures of less than 5°C can enhance resistance to several fungal pathogens in wheat, thought to be bought about by common biotic/abiotic stress pathways induced by exposure of plants to cold temperatures (Atkinson and Urwin, 2012; Ergon *et al*., 1998; Gaudet *et al*., 2011; Kuwabara *et al*., 2002). Therefore exposure of plants to the cold must be priming plant defence responses.

Thus there is evidence that low and high temperatures affect different aspects of plant defence, but little insight into how ambient temperature changes might influence plant defence pathways. Ambient temperature perception in plants is well recognised and plants have been shown to be able to detect temperature changes as little as 1°C (Argyris *et al*., 2005). There are many examples of developmental processes that take signals from ambient temperature cues such as flowering time, germination and circadian clock entrainment (Gimenez Luque *et al*., 2013; Kumar *et al*., 2012; Michael *et al*., 2008). Increased spread of viruses has long been correlated with low temperatures (Gerik *et al*., 1999; Zhang *et al*., 2012). It has previously been shown that RNA silencing plays a role in plant defence against viruses (Burton *et al*., 2000; Ratcliff *et al*., 2001). Temperature may be crucial to the virus induced silencing of plant defences since amounts of small interfering (si) RNAs increase with temperature (Chellappan *et al*., 2005; Szittya *et al*., 2003). In contrast, Zhong *et al* (2013) have more recently shown that an increase in temperature inhibits gene silencing. Lately, Kumar and Wigge (2010) revealed that H2A.Z-containing nucleosomes are responsible for regulation of the temperature transciptome in Arabidopsis. Further research might uncover a role for chromatin re-modelling in plant defence response to ambient temperature changes. In the study by Kumar and Wigge (2010), it was shown that *HSP70* transcript is strongly up-regulated when plants are shifted from temperatures of 12°C to 27°C, and that the transcript is expressed proportionally within that range, making it a useful tool for measuring a plant's ambient temperature perception in many species.

## 1.5 Rust resistance as a model for investigating effect of temperature on wheat defence

In the 1980s and 1990s, several studies were carried out to investigate the effect of temperatures on various QDR resistance genes in wheat. Most of these investigations were done with the interaction between wheat and various fungal rust pathogens (Dyck and Johnson, 1983; Gousseau and Deverall, 1987; Pretorius *et al*., 1994; Ramage and Sutherland, 1995). Rusts are among the most economically important and widespread diseases of wheat worldwide. There are three major rusts of wheat; stem rust caused by *Puccinia graminis* f. sp. *tritici*, leaf rust or brown rust caused by *P. triticina* and yellow or stripe rust caused by *P. striiformis* f. sp *tritici* (*Pst*). Yellow rust is currently the most economically important rust fungus in the UK, naturally preferring cooler, wetter conditions than the other rusts (Boyd 2005).

Dyck and Johnson (1983) identified several leaf rust QDR genes as sensitive to temperature whilst other QDR genes appeared insensitive, maintaining a constant resistance phenotype across temperature regimes, confirming that plants naturally have both temperature and non-sensitive resistance mechanisms. Ramage and Sutherland (1995) observed a difference in temperature sensitivity to different rust species from broad-spectrum QDR resistance conferred by *Lr20*/*Sr15.* The resistance conferred by this gene was more sensitive to temperature when challenged with *P. graminis* than when challenged with *P. triticina*. If a single gene product is responsible for resistance to both rust types, this indicates that temperature-sensitive resistance conferred by *Lr20/Sr15* must be specific to one type of rust. A similar differentiation in temperature sensitivity of *Sr9b* was observed when wheat plants were challenged with different isolates of *P. graminis*, emphasizing that differences can be seen at an isolate level in addition to a genus level (Gousseau *et al*., 1985). These observations support the hypothesis that temperature-sensitivity in plants is largely regulated by NB-LRR coding genes rather than other signalling components if *Lr20/Sr15* does encode two independent NB-LRR containing proteins. However if *Lr20/Sr15* encodes the same NB-LRR protein or something fundamentally different then the argument put forward by Zhu *et al* (2010) is not as straightforward as specific NB-LRR proteins being responsible for defence temperature sensitivity in plants. Gousseau *et al* (1985) argued that the temperature sensitivity of R genes must be due to the ability of the gene to

recognise the pathogen considering they saw differences between isolates but again, this hypothesis assumes that *Sr9b* is involved in recognition. Interestingly, Dyck and Johnson (1988) saw that resistance to leaf rust conferred by *Lr20* displayed a much stronger temperature sensitivity in one wheat cultivar compared to others, suggesting that other parts of the defence signal cascade may be affecting *Lr20*-conferred resistance differentially between lines. The literature reveals that there are clear differences between temperature-sensitivity in R genes and/or QDR genes, and that this temperature sensitivity may vary between different cultivars. As our understanding of resistance has advanced, further investigation of temperature sensitive defence genes in wheat is long overdue.

Yellow rust is a biotrophic fungal pathogen caused by *Pst* and is considered one of the most damaging diseases of wheat on a global scale. Indeed yield losses can be up 70% in extreme cases resulting of world losses of up to 20 million tonnes per annum (Clark, 2009; Chen 2005; Kosina *et al*., 2007). Yellow rust is becoming more prevalent, possibly due to the evolution of more aggressive isolates which have evolved to tolerate higher temperatures (Markell and Milus, 2008; Singh *et al*., 2011). In parallel, several major sources of wheat resistance to *Pst* have broken down in recent years (El Jarroudi *et al*., 2011; Rush, 2013; Clarke, 2012). These developments hasten the need to identify more effective and durable sources of resistance.

Several yellow rust QDR genes have also been identified in wheat as temperature-sensitive in that they perform better at higher or lower temperatures. Yellow rust resistance genes; *Yr36* and *Yr39* were initially designated high temperature adult plant (HTAP) genes due to evidence that higher temperatures are crucial to their function but can only confer resistance at later growth stages (Lin and Chen, 2007; Uauy *et al*., 2005). However, *Yr36* was later shown to confer superior temperature-dependent resistance at all growth stages when exposed to temperatures over 20$^{o}$C (Fu *et al* 2009). Cloning and sequencing of *Yr36 (WKS1)* revealed that the gene includes a kinase and a putative START lipid-binding domain, and that both are necessary to confer resistance to *Pst*. Further investigation revealed six alternative transcript variants designated *WKS1.1* to *WKS1.6*. Upon *Pst* challenge, *WKS1.1* was shown to be up-regulated, whereas *WKS1.2-6* transcripts were down-regulated. In addition, *WKS1.2–6* encode proteins with truncated START domains whereas *WKS1.1* encodes a complete WKS1 protein. Experiments done with temperature

cycles from a minimum of 10°C to a maximum of 35°C revealed a higher expression of *WKS1.1* transcript at higher temperatures relative to *WKS1.2-6* transcript. The authors postulated that the START domain may have the ability to bind lipids from *Pst* at high temperatures and that a subsequent change in confirmation of the domain could initiate a signal cascade leading to programmed cell death, revealing a possible reason for temperature sensitivity of *Yr36* resistance to *Pst* (Fu *et al*., 2009). However the genuine mechanism for the resistance conveyed by Yr36 is still to be determined.

In contrast to *Yr36*, evidence suggests *Lr34/Yr18* provides stronger resistance at lower temperatures, although the scope of how these resistance mechanisms relate to temperature is poorly-understood (Broers and Wallenburg, 1989; Plotnikova and Stubei, 2013; Pretorius *et al*., 1994; Rubiales and Niks, 1995). *Lr34/Yr18* is broad-spectrum and provides resistance against not only yellow rust, but also leaf rust and powdery mildew (Lillemo *et al*, 2008) although it is not known whether the temperature sensitivity of this gene is conveyed by challenge with all three pathogen types. Resistance gene *Lr34/Y18* is already well-established, with wheat cultivars containing this gene occupying more than 26 million hectares in various developing countries alone (Krattinger *et al*., 2009). Due to resistance of this gene being linked to cooler temperatures, it may be more suited to the UK climate, although it is not currently deployed here (Kolmer *et al*., 2008). *Yr36* has been introduced into many varieties worldwide through the introgression of the closely linked *Gpc-B1* gene (Kumar et al 2011; Randhawa *et al*., 2013; Tabbita *et al*., 2013), but its effectiveness and longevity in the field is yet to be determined. Several cultivars have been identified as containing unknown yellow rust temperature sensitive resistance genes, suggesting they are generally widely deployed in agriculture (Feng *et al*., 2011; Wan *et al*., 2000; Zhang *et al*., 2011). Webb *et al* (2010), propose that temperature sensitivity in QDR genes contributes to longer durability by reducing selection pressure on the pathogen population due to variability in disease levels between the hot and cold growing season. However, durability may also be due to the fact that they cannot be overcome by point mutations in the pathogen. Hypotheses about the evolutionary development of QDR-mediated defence at specific temperatures have been advanced. The wheat host may have evolved to take advantage of the warm weather conditions later in the growing season with resistance traits to win the battle against pathogen attack (Chen, 2013). In support of this, Wang *et al* (2009) propose that the ability to modulate defence expression could

provide enhanced resilience during the co-evolution between plants and their pathogens, due to evidence that some virulence factors are secreted most readily at temperatures below the optimum growth temperature for the plant (Smirnova *et al*., 2001). If expression of resistance is costly, then plants may have evolved temperature sensitive resistance genes to protect them against a particular pathogen within temperature ranges where they are at risk from invasion with the given pathogen (Figure 1.4, Alcázar 2011). However some temperature-sensitive resistances protect against multiple pathogens, so evolution cannot be due to this reason alone (Uauy *et al*., 2005; Krattinger *et al*., 2009). Importantly, Wang *et al* (2009) point out that there is no evidence that defence against necrotrophs is affected by temperature. Temperature modulation of resistance may therefore be a host strategy to deal with different types of pathogen with different virulence types attacking at different times. There is already evidence that plant resistance is tied into the circadian clock and that plants may be able to perceive an attack at dawn when pathogens are more likely to strike (Zhang *et al*., 2013; Wang *et al*., 2011). If this is the case then plants could also be using temperature to cue when to expect a challenge from a specific pathogen isolate or type. These hypotheses suggest that temperature-sensitive resistance genes could be useful in agriculture because they may be more durable. However before consideration for deployment of novel sources, more research is needed to evaluate how reliable they are in unpredictable weather conditions and their agricultural potential in the field.

Further exploration of environmental impact on plant defence will be essential to breeding management strategies, considering plant evolution can be controlled far more readily than pathogen evolution. Resistance to yellow rust research has been advanced in recent years, and with cloning of temperature sensitive QDR genes *Yr36* and *Lr34*, the interaction between wheat and *Pst* makes for an ideal system to study defence temperature sensitivity in wheat.

## 1.6 Effect of environment on plant pathogens

If resistance is temperature-sensitive and can be investigated and manipulated to manage disease outbreaks, then this will be a useful tool for dealing with climate change. However, resistance to some diseases is not available in modern wheat varieties and therefore has to

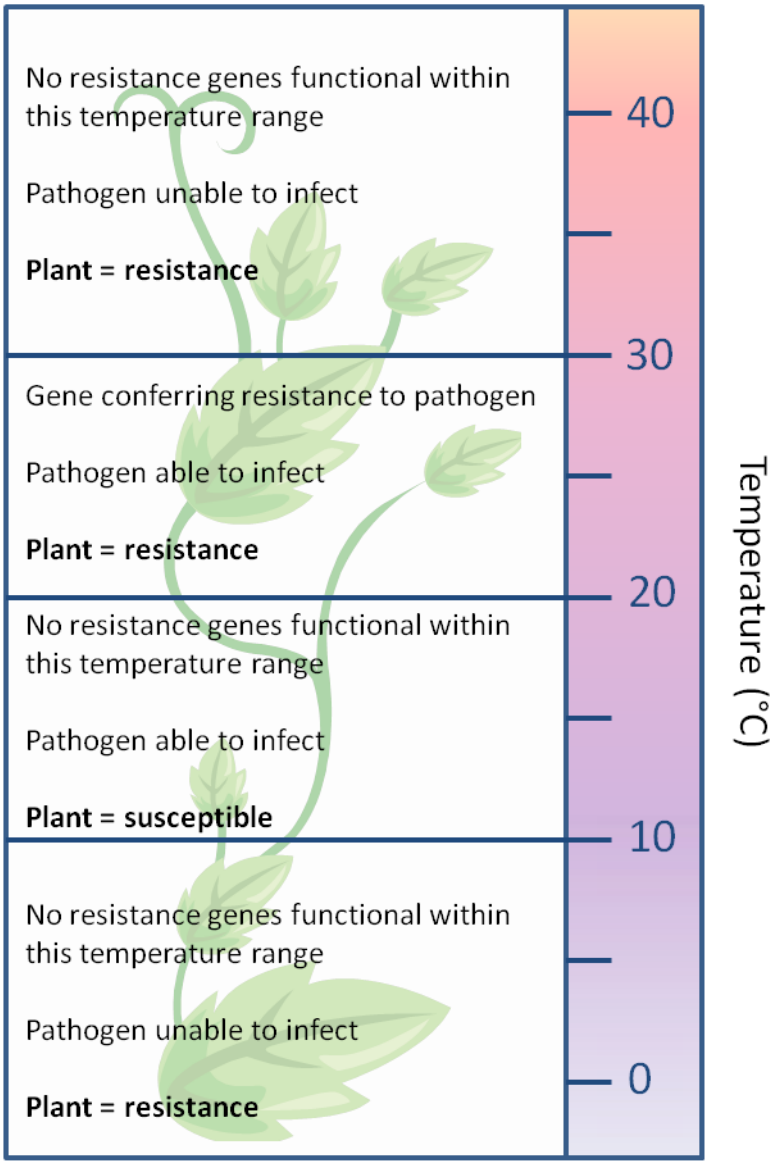



**Figure 1.4 Diagram to illustrate hypothesise by Wang *et al* 2009 and Alcázar 2011.** Authors hypothesised that temperature sensitive resistance genes may have evolved to protect the plant at some level in temperature ranges where specific pathogen isolates present in the population at the given time are able to infect.

be controlled by other agricultural management techniques such as crop rotation or fungicides. Take-all disease, which is caused by *Gaeumannomyces graminis* var. *tritici* (*Ggt*), is a rare example of a disease where there is little genetic variation in resistance amongst common wheat cultivars (Cook, 2003; Ennaifar *et al*., 2007; Kwak *et al*., 2009). There is a great deal of uncertainty about how climate change will affect soil pathogens although it is logical that they will increase due to predicted milder, wetter winters in the UK (Murphy *et al*., 2009). Development of take-all disease specifically is encouraged by warm winters and wet springs (HGCA, 2006). An example such as this is where it would perhaps be most useful to see how the environment affects disease outbreaks in order to prepare for climate change, especially considering a DEFRA funded project has already identified take-all as an increased risk in 2030 and 2050 using *UKCP09* (Thomas *et al*., 2010).

In addition to extreme, temporary temperature changes, it is the impact of subtle changes in mean temperature (of only a few degrees) on plant pathogens that necessitate investigation. Although seemingly insignificant, an increase of as little as 1°C in mean temperature has been shown to reduce rice yields by as much as 10% (Peng *et al*., 2004). Brennan *et al* (2005) investigated how a change of 4°C could affect *Fusarium* head blight disease in wheat. Findings suggested that 4°C was enough to see a difference in disease levels, but differences between cultivars and *Fusarium* isolates were not consistent, suggesting that temperature was also affecting plant resistance or susceptibility. The results also indicate that different disease-causing isolates vary in their temperature-sensitivity. Most pathogens have a temperature range which is optimal for their proliferation in the host. Within this range, disease severity may be affected but a threshold is met when pathogen proliferation starts to decline rapidly due to intolerance of high or low temperatures (Magarey *et al*., 2005). Temperature increases bought about by climate change will have a more severe affect on pathogens if they are maintained above optimum temperatures conducive to pathogen development and spread. Pathogens that occur nearer the equator are exposed to narrow temperature ranges, while pathogens at increased latitudes have evolved to tolerate more variable temperatures on a daily and seasonal basis. For this reason, the strongest effect of climate change is expected to be in the tropics due to pathogens in this region being already close to their tolerance threshold (Ghini *et al*., 2011). Increased overwintering of pathogens due to milder temperatures is likely to increase disease levels in the subsequent year, however this may be in conjunction

with increased decline of pathogens during summer months due to higher temperatures. There is evidence to suggest that pathogens are evolving to tolerate higher temperatures; for example, Milus *et al* (2006) found that new isolates of *Pst* in the US were more aggressive and tolerated higher temperatures than isolates obtained earlier. In support of this, a study by Peduto *et al* (2013) showed that *Erysiphe necator* which causes powdery mildew on grape plants was surviving at higher temperature than previously shown, indicating that new isolates are evolving to tolerate the high temperatures. It would be valuable to know whether pathogens can evolve to tolerate both higher and lower temperatures simultaneously, or whether adaption to one extreme comes with a cost of intolerance to the other. The argument that adaption to one environment reduced fitness to another has been presented by Kawecki and Ebert (2004). A study by Mboup *et al* (2012) shows that *Pst* isolates that are adapted to the South of France and therefore tolerant to higher temperatures, are still able to colonise in Northern France where temperatures are cooler, although as expected they colonised plants more successfully in the South. If pathogens generally become more aggressive when adapting to higher temperatures the outlook does not look good and may explain why we are seeing increasing threat from specific pathogens strains such as stem rust Ug99 isolates (Singh *et al*., 2011).

Based on this evidence, is it possible that instead of currently problematic diseases being replaced by newer ones better adapted to the new UK environment, will we face both new diseases and increasingly aggressive isolates of existing pathogens? This is unlikely due to the natural population balance and it is more plausible that the best adapted pathogens will win the battle against less adapted types, even if the newly-adapted types are more aggressive. It will be valuable to know which pathogens will win the race for development of fungicides, new cultivars and cropping systems.

## 1.7 Research aims

The ultimate aim of the work presented in the following chapters is to explore different approaches of investigating how climate change will affect diseases of wheat, with primary focus on temperature. The first approach involves looking at how defence against *Pst* in wheat is affected by current temperature variables to identify ways that this could be

manipulated to improve disease resistance that is resilient to changing temperatures. The second approach looks primarily at how increased temperature affects take-all disease. These approaches will be critically analysed to determine the best way to prepare for climate change impact on plant diseases.

### 1.7.1 Effect of temperature changes in defence against *Pst*

Although plant defence gene function is clearly affected by environmental signals, very little work has been done in trying to understand precise environmental conditions and sustained condition periods required to maintain function. It is apparent that some R gene resistance mechanisms are dependent on temperature, but it is not known how long the plant must be maintained in an environment and whether a temperature threshold exists for resistance to be sustained. Also, previous studies have mostly focused on how average temperature affects resistance but this research is primarily concerned on how a change in temperature or continual temperature variation is affecting resistance. Concerns over control of yellow rust, and the recent characterisation of temperature-sensitive *Pst* resistance make this patho-system ideal for the investigation.

*Yr36* has been shown to be temperature sensitive, but its potential in UK agriculture has yet to be demonstrated, and is the starting point for this investigation. It has been shown that *Yr36* confers resistance if day temperatures are above 20°C (Uauy *et al.,* 2005; Fu *et al*., 2009). Initial work addresses the performance of *Yr36* at different and changing temperatures. For example, if plants are grown in one temperature regime and transferred to the later, how long is the effect of *Yr36* sustained? Qayoum and Line (1985) reported that HTAP conferred resistance was not sustained when plants were returned to lower temperatures, however this study was done using lines with unknown HTAP resistance so it is difficult to draw any general conclusions. During the investigation, an effect of temperature change on resistance was established, although this was shown to be independent of *Yr36*. Subsequent work aimed to explore the basis for this, addressing hypotheses that may account for the observation.

### 1.7.2 Impact of climate change on the incidence of take-all

The other thread of this thesis focuses on looking at how temperature affects disease levels of take-all which cannot be controlled by breeding for resistance. The aim is to combine historical datasets for disease incidence with meteorological records to identify important factors in disease spread. The work will assess to what extent the data from different regions can be combined to make predictions about whether future climate will influence spread. Moreover, the thesis will determine how the results could be combined with climate model UKCP09 to accurately predict regions that will experience an increased threat from take-all with climate change.

Finally, the methodological approaches throughout the thesis will be critically assessed, as the overall aim of this thesis is to explore different ways in how best we can prepare wheat from the threat of disease under climate change.

# Chapter 2: Materials and Methods

## 2.1 Materials

### 2.1.1 Plants

Wheat varieties (*Triticum aestivum*) were obtained from various sources and summarised in Table 2.1.

### 2.1.2 Bacterial Strains

*Pseudomonas syringae* pv. *oryzae* strain *Por*36_1 was obtained from Dr Kee Sohn, The Sainsbury Laboratory, Norwich, UK and was originally described by Hwang *et al*., (2005).

### 2.1.3 Fungal isolates

Yellow rust *(Puccinia striiformis* f. sp. *tritici)* race 08/21 was isolated in 2008 from wheat cultivar Solstice and race 08/11 was isolated from cultivar Warrior. Both isolates were provided by NIAB, Cambridge, UK

Take-all (*Gaeumannomyces graminis var. tritici*) strains were isolated at Rothamsted Research farm, Harpenden, UK in 2010.

Powdery mildew (*Blumeria graminis f. sp. tritici)* isolate JIW48 was provided by Margaret Corbitt, John Innes Centre.

Spores of *Fusarium culmorum* isolate Fu 42 were provided by Andy Steed, taken from the John Innes Centre Facultative Pathogen Collection.

### 2.1.4 Chemicals and antibiotics

All chemicals were from Sigma Aldrich (St. Louis, MO, USA) unless otherwise stated. Antibiotics were as follows: ampicillin (Sigma Aldrich), nystatin (Melford Laboratories Ltd)

**Table 2.1 Wheat lines/cultivar used in experiments**

| Wheat line/ cultivar | Source | Notes |
|---|---|---|
| Alpowa | UK National Germplasm Collection, John Innes Centre | US spring wheat with pedigree Fielder//Potam 70/3/Walladay x 2/4/Walladay/Potam 7 |
| Anza | Cristobal Uauy, John Innes Centre | US spring wheat with pedigree Lerma Rojo x Norin 10 Bravor |
| Cerco | Maraget corbitt, John Innes Centre | US winter wheat NP 98*WA 4765 |
| Claire | Lesley Boyd, John Innes Centre | European winter wheat with pedigree Wasp x Flame |
| Hereward | RAGT Seeds Ltd, Essex, UK | European winter wheat with pedigree Norman 'sib' x Disponent |
| Sappo | UK National Germplasm Collection, John Innes Centre | European spring wheat |
| Shamrock | Nikolai Adamski, John Innes Centre | European winter wheat from pedigree COMPL TIG 323-1-3m x CWW 4899/25 |
| Solstice | Rosemary Bayles, NIAB | UK bread wheat with pedigree Rialto x Vivant |
| UC1401 | Cristobal Uauy, John Innes Centre | US hexaploid spring breeding line derived from the cross Tadinia x Yecora Rojo |
| UC1041 + *Yr36* | Cristobal Uauy, John Innes Centre | Near isogenic line to UC1041 containing resistance gene *Yr36* |

rifampicin (Duchefa, Haarlem, The Netherlands) and streptomycin (Fischer Scientific, Leicestershire, UK).

### 2.1.5 Pathogen culture media and buffers

**KB (Kings B Medium, King *et al*., 1954)**

Formula per 1 litre de-ionised water:

20 g Protease Peptone

pH 7.2

For solid 15 g M agar

**Mildew culture medium (Boyd *et al*., 1994)**

Formula per 1 litre de-ionised water:

0.1 g benzamidazole

5 g M agar

**PDA**

Formula per 1 litre de-ionised water:

39 g Potato dextrose agar

**PDB**

Formula per 1 litre de-ionised water:

12 g Potato dextrose broth

**Sand-maize meal medium**

Formula per 1 kg

900 g Sand

100 g Maize meal

200 mL sterile de-ionised water

**TE**

Formula per 1 litre de-ionised water:

1.21 g Tris

**V8™ (Campbell Soup Co.) medium**

Formula per 1 litre de-ionised water:

200 mls V8™ juice

18 g M agar

**Water agar**

Formula per 1 litre de-ionised water:

30 g agar

### 2.1.6 Plant growth media

**Cereal mix**

40% Medium Grade Peat

40% Sterilised Loam

20% Horticultural Grit

1.3kg/m³ PG Mix 14-16-18 + Te Base Fertiliser

1kg/m³ Osmocote Mini 16-8-11 2mg + Te 0.02% B

Wetting Agent

3kg/m³ Maglime

300g/m³ Exemptor

## 2.2 Methods

### 2.2.1 Plant growth

Seed surface sterilisation:

When required, seeds were sterilised by washing seeds in bleach containing approximately 1% sodium hypochlorite for 2 mins, followed by washing with 70% ethanol for 1 min.

For disease tests:

Seeds were sown directly into cereal mix in plantpak (p)15 cells, (p)24 cells or 1 litre pots depending on final experimental growth stage required. Watering was as required.

For seed bulking:

Seeds were placed on damp filter paper and kept in the dark for 6-8 weeks to allow for vernilisation requirement (when required). Seeds were then transferred to 1 litre containing cereal mix and grown under glasshouse conditions.

### 2.2.2 Temperature regimes

CE facilities:

After sowing plants for controlled environment experiments were grown in CERs or CE cabinets from Sneijder (Tilburg, The Netherlands) or Sanyo (Gallenkamp PLC, UK). The various regimes used are outlined in each chapter.

CE monitoring:

CE room or cabinet humidity and temperature were frequently monitored using SL54TH® data loggers (Signatrol Ltd, Tewkesbury, UK). Light level consistency between cabinets was also checked using a Quantitherm light/ temperature sensor (Hansatech Instruments Ltd, Pentney, kings Lynn, UK). Controlled environment facilities were often changed between

experimental repeats to account for other environmental variables due to the nature of the experiments.

### 2.2.3 Pathogen isolation and maintenance

#### 2.2.3.1 Yellow rust (*Pst*)

Bulking and maintenance:

Spores were maintained under liquid nitrogen vapour and Solstice plants were used to bulk spores as required. Plants were grown for 2 weeks under glass house conditions. Plants were sprayed with $H_2O$ containing Tween20 ® (0.01 % v/v) to encourage spore attachment and germination, then inoculated using a spore/talc mixture at a ratio of 1:1. Plants were incubated in darkness for 24 hrs at 12°C, 100% humidity. Plants were then returned to the glasshouse chamber. Spores were harvested from 14 days and either used immediately or stored in liquid nitrogen vapour until required.

#### 2.2.3.2 Take-all (*Ggt*)

Isolation:

*Ggt* strains were isolated from various wheat plants in fields collected from Rothamsted Research farm. Plant roots were washed for 2 mins in 100% ethanol followed by a wash with 1% sodium hypochlorite with a drop of Tween® 20. Roots were then rinsed three times in sterile water and cut into small pieces and placed on PDA containing 100 µg $mL^{-1}$ streptomycin 100 µg $ml^{-1}$ ampicillin. Plates were incubated in darkness at 20°C. After 4 days, plugs of mycelium were taken from the root sections and transferred to fresh PDA. After a further 9 days, mycelium was transferred to conical flasks containing PDB and shaken in darkness at 20°C for 5-7 days. Mycelium in a liquid culture was removed from the flask and drained using sterile filter paper and divided for use in either re-inoculating fresh root tissue or DNA extraction. The fresh mycelium was mixed with sterile vermiculite and placed in Falcon tubes into which sterile Herewood seeds were placed and incubated at 20°C for 2 weeks. All roots grown in different inoculum sources made up from various isolates developed take-all disease-like symptoms.

DNA extraction and sequencing:

Mycelium for DNA extraction was freeze dried for two days before being ground using the same procedure as in section 2.2.5. DNA was extracted using DNeasy Plant Mini Kit (Qiagen) and subsequently amplified by PCR using ITS primers (1a and 1b in Table 2.2) and cycling conditions detailed in Daval *et al* (2010). PCR products were used as a template in the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturers instructions. Sequencing analysis was performed by Genome Enterprise Ltd (John Innes Centre). All sequences were most closely matched to *Ggt* species when a BLAST (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi) search was performed.

Maintenance:

*Ggt* strains were maintained under oil submersion at 4°C. When required, strains were sub-cultured onto PDA plates and plugs were taken from the edge of the new colony to reduce growth restriction from oil.

#### 2.2.3.3 Powdery mildew (*Bgt*)

Maintenance:

The isolate was maintained on detached wheat leaves of the cultivar Cerco and transferred to fresh leaves every 2-3 weeks. Leaves were inoculated using an assay adapted from Boyd *et al* (1994) where leaf tissue was cut from plants and placed in boxes containing mildew culture media and spores from previous leafs were tapped onto new leaves.

#### 2.2.3.4 *P. syringae*

Antibiotic resistance selection and maintenance:

*P. syringae* isolate *Por*36_1 was initially screened for colonies with rifampicin resistance. Resistant bacteria was maintained as a glycerol stock (15%) and streaked onto KB agar plates containing 50 mg/l rifampicin and 25 mg/l nystatin no more than 24 hrs before needed.

#### 2.2.3.5 *Fusarium culmorum*

Maintenance:

*F. culmorum* conidia were suspended in $ddH_2O$ and stored at -20°C until required.

### 2.2.4 Microscopy

Tissue preparation and staining:

Leaf segments were harvested at various time points post inoculation and prepared for microscopy using a method adapted from Ayliffe *et al*., (2011). For removal of chlorophyll, samples were left to clear overnight in 12 mL of 1 M KOH with 2 µL of Tween20 ® at 37°C. The tissue was rinsed three times in 50 mM Tris at pH 7.5, followed by staining with WGA-FITC at 1 mg $mL^{-1}$ (made up with 50 mM Tris) for 1 hr.

Slide preparation and viewing:

The tissue was mounted on a slide and observed under fluorescent light (465-495nm > 515-555nm) using a fluorescence microscope (Nikon 800 Eclipse; Nikon Precision Europe GmbH, Langen, Germany) at 10X or 20X magnification. Images were captured using a Pixera Pro ES600 (World Precision Instruments, Stevenage, UK).

### 2.2.5 Sampling tissue, storage and grinding

Leaf tissue was flash frozen in liquid nitrogen and stored at -80°C prior to RNA or DNA extraction. Tissue was ground by adding two 5 mm cone grinding balls (Retsch®) and shaken for 2 mins at an oscillation speed of 50 1 $s^{-1}$ using a tissue lyser LT (Qiagen).

### 2.2.6 Wheat Genotyping

DNA was extracted from plant tissue of seedlings of near isogenic lines UC1041 +/- *Yr36* by macerating leaf tissue in Eppendorf ® tubes with a mini pestle then adding 300 µL of buffer made up with 200 mM Tris HCl pH7.5, 250 mM NaCl, 25mM EDTA with 0.5% SDS. Leaf tissue was further ground and vortexed then spun at 13 rpm for 1 min. 300 µL of solution was transferred to a new tube containing an equal amount of isopropanol and substrate was mixed and incubated at room temperature for 2 mins. Samples were spun again at 13

rpm for 5 mins, washed with 75% ethanol, dried and re-suspended in 100 µL TE buffer. 1 µL of a 1:20 dilution of the DNA was used in a 20 µL PCR reaction with 1 µL of each primers 3a and 3b and 3c from Table 2.2 at 10 µM, 1 µL of dNTPs, 2 µL of PCR reagent mix (Qiagen), 13.8 µL of dH2O and 0.2 µL of Taq polymerase (Qiagen). Samples were also amplified with a reference gene primer from Table 2.3 to confirm that the reaction had worked for the -*Yr36* line. Cycling conditions were as follows: an initial denaturation step of 94 °C for 4 mins, followed by 40 cycles of 94°C for 30 secs, 60 °C for 30 secs and 72 °C for 1 min; followed by extension at 72°C for 10 mins. 6 X loading buffer (0.1 M EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol, 30% glycerol) was added to nucleic acid samples before they were run on an agarose gel containing ethidium bromide to check for the presence or absence of the *Yr36* gene.

### 2.2.7 RT-qPCR

Isolation of plant RNA:

Tissue was ground using the method detailed in section 2.2.5. RNA was extracted from a maximum of 100 mg of leaf tissue using RNeasy Plant Mini Kit (Qiagen) or TRI-Reagent® (Sigma-Aldrich) according to manufacturer's instructions excluding the use of β-mercaptoethanol.

First strand cDNA synthesis:

Prior to cDNA synthesis, contaminating DNA was removed from RNA samples by treating with Ambion® Turbo DNA-free™ (Life Technologies Ltd) according to the manufacturer's instructions. RNA was quantified using a Picodrop® spectrophotometer (Picrodrop Ltd, Cambridge, UK), discarding any samples that did not fall between 1.8 and 2.0 of the $OD_{260}/OD_{280}$ ratio. Samples were adjusted to the same concentration to allow a total of 1 µg of RNA for the cDNA synthesis. First-stand cDNA was synthesised from RNA using the SuperScript™ III First Strand Synthesis System (Invitrogen) in RT-PCR. 1 µg of total RNA with both oligo dT and random primers in equal measures was used in a 20 µL reaction following the supplier's instructions. To determine whether there was any RNA remaining in the end product, a control for each experiment was formed by following the supplier's instructions with the exception of adding dH2O instead of SuperScript™ III. The control was run alongside samples in the PCR reaction.

Quantitative RT-PCR (qRT-PCR) and qPCR:

5 µL of a 1:20 dilution of the cDNA or DNA was used in a 20 µL PCR reaction with 0.4 µL of each primer at 10 µM, 10 µL of SYBR® Green JumpStart™ Taq Readymix (Sigma-Aldrich) and 4.2 µL of dH2O. Two PCR replicates of each sample were run using the DNA engine Opticon 2 Continuous Fluorescence Detector (MJ Research Inc, Alameda, CA, USA). Cycling conditions were as follows: an initial denaturation step of 95 °C for 4 mins, followed by 40 cycles of 94°C for 30 secs, 60 °C for 30 secs and 72 °C for 30 secs; followed by extension at 72°C for 10 mins.

Quantitative qRT-PCR analysis:

Where possible, all samples were run on the same plate with reference genes for each experiment. When this wasn't possible, amplification of each gene was done separately. A melt curve analysis was performed (65-95°C) to distinguish PCR products from amplification artefacts and data were analysed using Opticon Monitor analysis software v3.1 (MJ Research Inc). The average Ct (threshold cycle) was calculated from two technical replicates of each sample and the RNA transcript levels were normalized to the geometric mean of the most (or two most) stable reference genes in the given experiment (see section 2.2.8 for selection method). Normalised expression data were plotted directly or relative expression was calculated from normalised expression ratios (Pfaffl *et al*., 2001).

### 2.2.8 Primers

See Tables 2.2 and 2.3 for details and origin of primers used in various experiments.

Primer design:

Primers were designed using sequence data from the location specified in Table 2.2 and 2.3 using primer3 v4.0 (Rozen and Skaletsky, 2000). Primer efficiency was calculated for each primer using a classical calibration dilution curve and slope calculation (http://www.gene-quantification.info/).

Reference gene selection and data analysis:

For each experiment, up to five reference genes were tested to determine the most stable in the given treatments. The stability of reference genes was tested using genorm v3.5 (http://medgen.ugent.be/~jvdesomp/genorm/; Vandesompele *et al*., 2002).

**Table 2.2 Primers used in various PCR throughout thesis**

| Primer no. | Gene | Primer direction | Primer sequence | Primer reference |
|---|---|---|---|---|
| 1a | ITS1 | Forward | CGAACTCGGTCGTTTAGAGG | Daval *et al*., 2010 |
| 1b | | Reverse | TTCTTCGCTTATTGATATGC | Bryan *et al.,* 1995 |
| 2a | Avenacinase gene from *Gaeumannomyces graminis var .avenae* | Forward | GCCTCCATTGAGGCTGGTCTG | Designed using sequence publish in Rachdawong *et al* (2002) |
| 2b | | Reverse | ACCCTCCGGGAGGAGAGTGTG | as above |
| 3a | Yr36 | Forward | CACAAGTACAATACCTTATGAAGATGG | Fu *et al*., 2009 |
| 3b | | Reverse | CCTGAGCCCAGCAATACT | As above |
| 4a | HSP70 | Forward | GACACTCACCTGGGTGGA | Designed by Laura Dixon, John Innes centre |
| 4b | | Reverse | CGCCGTCCGGAGACGACGC | as above |
| 5a | Chitin elicitor binding protein (CEBiP) | Forward | TCGCCTACATCGTCGACGGC | Designed by Henk-jan Schoonbeek, John Innes centre |
| 5b | | Reverse | GTGTAGGTGCCGTTCGGGAG | As above |
| 6a | FLAGELLIN SENSITIVE2 (FLS2) | Forward | CGACGTCTCTGACGAATCTTG | Designed by Henk-jan Schoonbeek, John Innes centre |
| 6b | | Reverse | GTTGTGGATGACGAGCTTCTG | as above |

**Table 2.3 Primers to amplify wheat reference genes in qRT-PCR.** Unnamed reference genes were identified as potential candidates in a screen by Long *et al.,* 2010

| Gene | Primer direction | Primer sequence | Primer reference |
|---|---|---|---|
| Elongation factor-1 alpha (EF-1α) | Forward | TGGTGTCATCAAGCCTGGTATGGT | Provided by Graham McGrann, John Innes Centre |
| | Reverse | ACTCATGGTGCATCTCAACGGACT | as above |
| HvGAPDH | Forward | CCTTCCGTGTTCCCACTGTTG | Provided by Graham McGrann, John Innes Centre |
| | Reverse | ATGCCCTTGAGGTTTCCCTC | as above |
| Ubiquitin | Forward | CCTTCACTTGGTTCTCCGTCT | Provided by Graham McGrann, John Innes Centre |
| | Reverse | AACGACCAGGACGACAGACACA | as above |
| Microarray Sequence ID: Ta.27922.1.S1_at | Forward | TGACCACCCTCAGCCTGAAA | Designed using sequence from PLEXdb |
| | Reverse | GCCATGTTCCAGCTCCCTCT | as above |
| Microarray Sequence ID: Ta.7800.1.A1_at | Forward | CCTGAGCAGACAATGGGAGAGA | Designed using sequence from PLEXdb |
| | Reverse | AATGCAAAAAGGCACCAGCA | as above |

### 2.2.9 Data analysis

Data were analysed using the statistical package Genstat for Windows, release 12 (VSN international, Hemel Hempstead, UK) or Excel (Microsoft Office 2007). Details on specific tests are provided in each chapter. Prior to statistical analysis, all data were checked for normal distribution using the Genstat inbuilt “model checking” function. Data that were not normally distributed were transformed using the method detailed in each section. Residuals from analysis of variance were also checked for normal distribution.

# Chapter 3: Exploring the nature of temperature sensitive resistance to yellow rust in wheat

## 3.1 Aim:

The work described in this chapter was undertaken to investigate the nature of temperature sensitive gene *Yr36* to assess its potential in UK agriculture.

## 3.2 Introduction

The nature of yellow rust and the importance of incorporating new sources of resistance into the gene pool have been outlined in chapter one. Included are several yellow rust resistance genes that have shown temperature sensitivity in wheat. HTAP *Yr36* was mapped in *Triticum turgidum ssp. dicoccoides* to chromosome 6B (Uauy *et al*., 2005). The gene was later cloned and found to include a kinase and a putative START lipid-binding domain which increased plant resistance at higher temperatures (Fu *et al*., 2009). *Yr36*-mediated resistance is effective when day temperatures are maintained at 25°C or reach a maximum of 35°C. However the resistance is not effective when day temperatures are maintained at 20°C, suggesting that exposure to temperatures above 20°C are crucial for it to function (Uauy *et al*., 2005). *Yr36* is not commercially deployed at present but has been introduced into many varieties worldwide through the introgression of the closely linked *Gpc-B1* gene (Kumar *et al*., 2011; Randhawa *et al.,* 2013; Tabitta *et al.,* 2013). However it is unclear whether the alleles would be of value in the UK as temperatures are usually cooler than in other wheat growing areas of the world. The temperature sensitive nature of HTAP resistance gene *Yr39* was also briefly investigated after initial observations with *Yr36* (Lin and Chen, 2007). *Yr39* confers a similar level of resistance to *Yr36* and is also a major effect QTL (Coram *et al*., 2008).

This chapter includes an investigation of early stage *Pst* colonisation by microscopy. *Pst* can infect wheat plants at any growth stage provided the tissue is green (Chen 2005). Urediospores germinate on the wheat leaf surface, forming a germ tube that enters the plant through the stomata. Once inside, a sub-stomatal vesicle (SSV) is formed within the stomatal cavity from which infection hyphae form. A haustorial mother cell is formed at the end of each infection hyphae upon contact with a plant mesophyll cell. An infection

peg breaches the plant cell wall forming a fungal feeding structure, known as a haustorium, within the cell. Further hyphae develop from the infection hyphae and proliferate throughout the leaf (Hovmoller *et al*., 2011). A graphical representation of how *Pst* invades host tissue is shown in Figure 3.1. Approximately two weeks after the pathogen has entered the plant cells, visible symptoms can be seen either as chlorosis or as pustules forming on the leaf surface in susceptible wheat cultivars. In cultivars containing specific R genes, *Pst* is able to enter the plant cells and form haustoria, but HR-triggered cell death prevents further infection spread usually within 48 hrs (Wang *et al*., 2013b). This response can usually be detected visually as necrotic tissue begins to develop around the area of infection. In contrast, although *Pst* can form pustules in cultivars with QDR, symptoms are less severe and develop later than those on susceptible varieties (Krattinger *et al*., 2009; Qamar *et al*., 2012; Uauy *et al*., 2005).

Investigations on temperature-sensitive resistance genes have explored which temperatures are important for gene function, but little attention has been paid to the longevity of gene function when plants are exposed to a temperature, crucial for function, for a set time period and then removed from it. Temperature regimes based on those used in Uauy *et al.,* (2005) and Fu *et al* (2009) were used to address this question. Results reveal that resistance conferred by *Yr36* in hexoploid breeding line UC1041 is compromised by a change in temperature rather than prolonged exposure to a lower temperature as previously reported. Findings reveal a background-sensitivity to temperature changes in UC1041 which was further investigated. Results uncover when a change in temperature in UC1041 is important and when it can first be detected microscopically. Results also show that sensitivity to temperature changes may vary between cultivars, so could inform breeding to create wheat varieties with more consistent *Pst* resistance under varying temperatures.

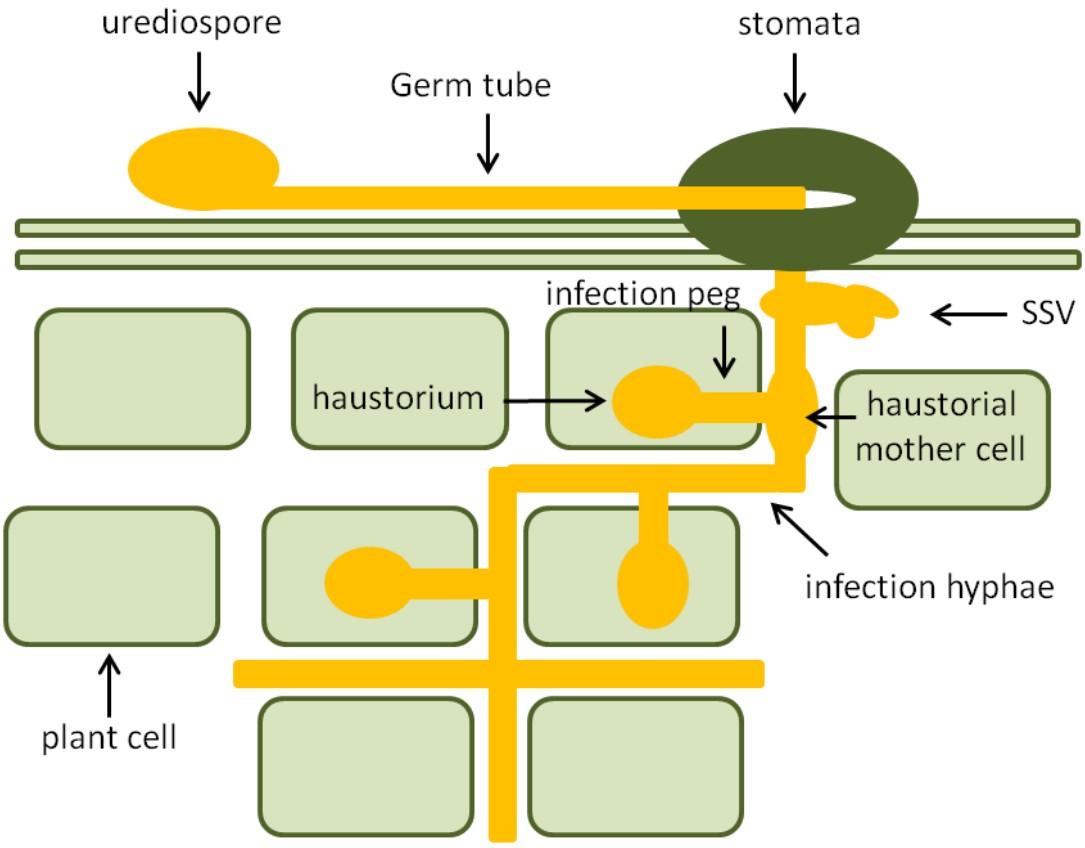



**Figure 3.1 Graphical representation of *PST* invading a host tissue.** Progression of successful *Pst* invasion of a susceptible host. Host tissue is shown in green and *Pst* tissue in yellow.

## 3.3 Methods

### 3.3.1 Plant and pathogen material

US wheat breeding line NILs UC1041 +/- *Yr36* and cultivars Alpowa (due to the variety containing *Yr39*), Shamrock, Sappo and Solstice were used in these experiments. None of these cultivars had any known R genes for *Pst* isolate 08/21 which was the isolate used in all experiments. See section 2.1 for details on plant and pathogen material.

### 3.3.2 Plant growth conditions

Seeds were sown directly into 1 litre pots for growing to flag leaf stage (Zadoks scale 47), or in P15 seed trays for seedling (Zadoks scale 13-14) assays (Zadoks *et al*., 1974). In the case of Alpowa only, plants were grown to stem elongation stage (Zadoks scale 30). Plants were grown in Controlled Environment Rooms (CERs) with an 8 hr/16 hr dark/light cycle, a constant 80% relative humidity and a light intensity of approximately 350 µmol $m^{-2}$ $s^{-1}$. The two diurnal temperature regimes (based on those used in studies by Uauy *et al*. (2005), and Fu *et al*., (2009) were 12/18°C and 12/25°C (day temperature being the only difference between them). To reduce the effect of non-temperature variables influencing results, different CER facilities were used in each experiment for adult UC1041 plants. To synchronise growth stage for inoculation of flag leaves, plants were sown one week earlier in the 12/18°C regime. For assays on wheat seedlings, plants were sown one to two days earlier in the 12/18°C regime, depending on the cultivar.

### 3.3.3 Inoculation of plants

Inoculations were carried out on flag leaves of adult plants, and the newest fully-developed leaf of seedlings. Plants were always inoculated with *Pst* urediospores within 1 hour before the end of the light period. A 4 cm (seedlings) or 5 cm (adult plants) region of the adaxial surface of the leaf was defined and urediospores were applied with a fine brush containing a 1:8 spore/talc combination (young plants) or 1:4 (adult plants). The leaf surface was then sprayed with $H_20$ containing Tween20 ® (0.01 % v/v) to encourage germination. In UC1041 NIL comparison experiments and inoculation of cultivar Alpowa, the same procedure was

used to apply spores but whole leaves were inoculated. Plants were placed in a dew chamber at 12°C in darkness for 22 hrs before they were either returned to the original temperature regime, or transferred to the new regime. At 18 days post inoculation (dpi), the same 4-5 cm region (or the whole leaf for UC1041 NIL comparisons) was used to determine the pustule cover in the given area, taken as the percentage of leaf tissue (independent of chlorosis or necrosis) with sporulating uredinia.

### 3.3.4 Transfer of plants

Plants were transferred from one temperature regime to another by physically moving pots from one CER to the other, pre or post incubation with the pathogen. The majority of experiments involved transferring plants after incubation with the pathogen in the dew chamber. For experiments when plants were transferred from one temperature regime to the other pre inoculation, they were moved at the beginning of the dark period.

### 3.3.5 Microscopic analysis of *Pst* development

Inoculated seedlings of UC1041 plants were sampled at 1, 3, 6 and 8 dpi. The 4 cm inoculated region of the leaf was harvested and prepared for microscopy using the method outlined in section 2.2.4. Samples from early time points were examined for both spore germination rates and ability of germinated uredospores to form SSVs. Later time points were scored by measuring the size of internal fungal structures (µm) and abundance of hyphae in up to 50 fields of view measuring approximately 0.28 $mm^2$**.**

### 3.3.6 Photosynthesis measurements

Gas exchange measurements were taken in flag leaves of adult UC041 plants using a portable photosynthesis system Li-COR LI-6400 model (Lincoln, NE, US), at 1 day, 2 days and 8 days post plants being changed between temperature regimes (in the absence of pathogen challenge). Measurements were taken by inserting a section of the leaf into a small micro-environment which was set to the temperature at which the chamber was programmed to an RH of 80% and an irradiance of 1200 µmol $m^{-2}$ $s^{-1}$. Assimilation rates and

internal $CO_2$ concentrations were recorded which were used to generate *A*-$C_i$ curves at a range of external $CO_2$ concentrations. Calculations were in accordance with Farquhar's photosynthesis model (Farquhar *et al*., 1980) with ambient $CO_2$ at 380 mmol mol $^{-1}$.

### 3.3.7 Statistical design and data analysis

Where possible, four or more individual plant replicates and more than one experimental repeat were used to calculate means and standard error unless otherwise specified. Data were analysed using the statistical package Genstat for Windows, release 12 (VSN international, Hemel Hempstead, UK). Percentage infection scores were transformed using a LOGIT$^+$ transformation to obtain near normality (Powell *et al*., 2013).

$$LOGIT^{+} = \log_n \left[ \frac{(Pi + (\min Pi + 0.25))}{((\max Pi + 0.25) - Pi)} \right]$$

Where $Log_n$ is natural logarithm and *Pi* is percentage pustule cover. A general linear regression model was used on the transformed data and outputs from the model provided predicted means where multiple experiments were performed. The effect of temperature regime and experiments was accounted for in the model. *Pst* microscopy data were also analysed with a general linear regression model using a LOGIT + transformation for percentage data. An unpaired *t* test was used to determine differences between treatments from the photosynthesis measurements.

## 3.4 Results

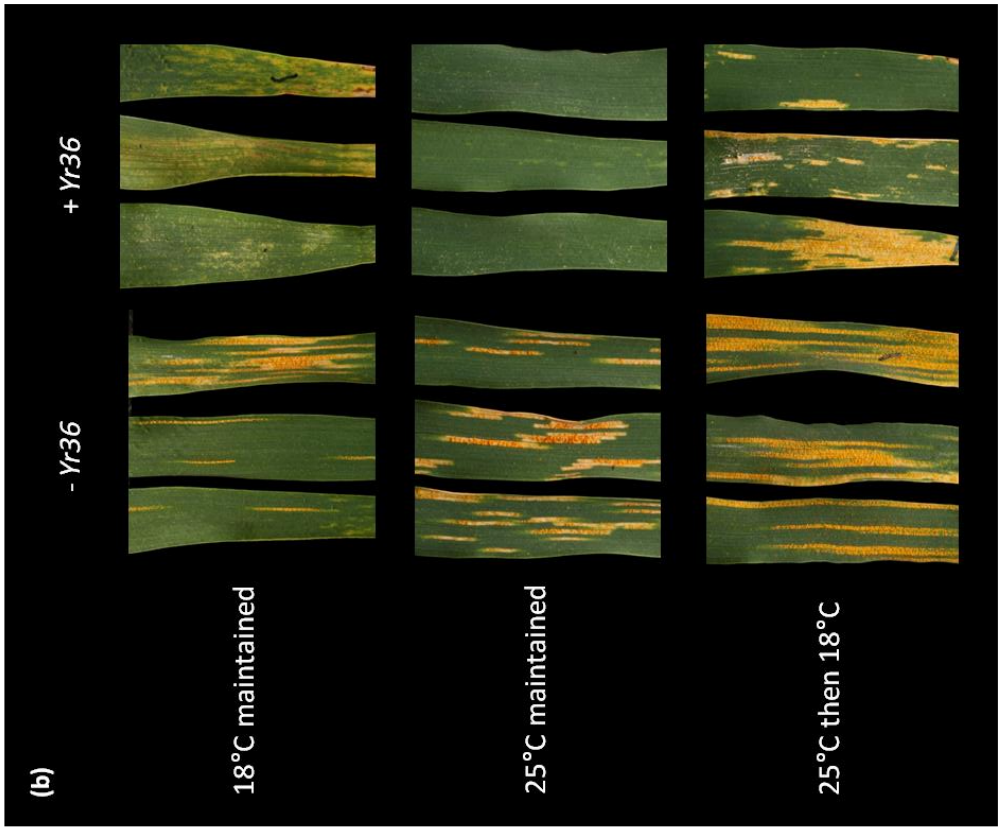
### 3.4.1 A reduction in day temperature compromises resistance to *Pst* in UC1041 independently of *Yr36*

*Yr36* conferred almost complete resistance to *Pst* in adult UC1041 *+Yr36* when plants were maintained in either the 12/18°C or the 12/25°C temperature regimes pre and post

inoculation. However *Yr36* mediated resistance was significantly reduced in plants originally grown at 12/25°C then transferred to 12/18°C, as pustule coverage was significantly higher ($P < 0.01$, Figure 3.2a,b). As expected, UC1041 -*Yr36* plants were less resistant than UC1041 *+Yr36*, and there was no significant difference in disease levels between plants which were maintained at 12/18°C or 12/25°C pre and post-inoculation (Figure 3.2a,b). Similar to results seen for UC1041 *+Yr36*, resistance in UC1041 -*Yr36* plants was significantly reduced when plants were transferred from the 12/25°C regime to 12/18°C following infection, with leaf pustule coverage increasing up to two fold ($P < 0.01$). Changing temperatures therefore affects resistance in both UC1041 NILs independent of the presence of *Yr36*.

### 3.4.2 An increase or decrease in temperature affects *Pst* resistance up to 8 days pre-inoculation in UC1041 -*Yr36*

Further investigations were carried out to characterise the temperature-sensitive nature of the UC1041 background. As previously observed, there was no significant difference in percent pustule coverage of plants maintained pre and post inoculation either 12/18°C or 12/25°C (Figure 3.3a,b), whilst plants transferred from the higher to the lower temperature regime were less resistant ($P < 0.01$). Conversely, plants grown at 12/18°C and then transferred to 12/25°C post inoculation were more resistant with significantly lower pustule levels ($P < 0.001$, Figure 3.3a,b). Although the relative disease levels varied between experiments, the trend in adult UC1041 -Yr36 plants remained consistent. Similar experiments were performed with plants transferred from one temperature regime to the other up to 8 days before inoculation. There was no significant difference in pustule levels between plants transferred from 12/18°C to 12/25°C at the time of inoculation compared to those transferred 1, 2, 3, 5 or 8 days prior to inoculation (Figure 3.4a,c). Adult plants transferred from 12/25°C to 12/18°C had increased numbers of pustules, again regardless of whether they were transferred at the time of inoculation or after 1, 2, 3, 5 or 8 days pre-inoculation (Figure 3.4b,d).



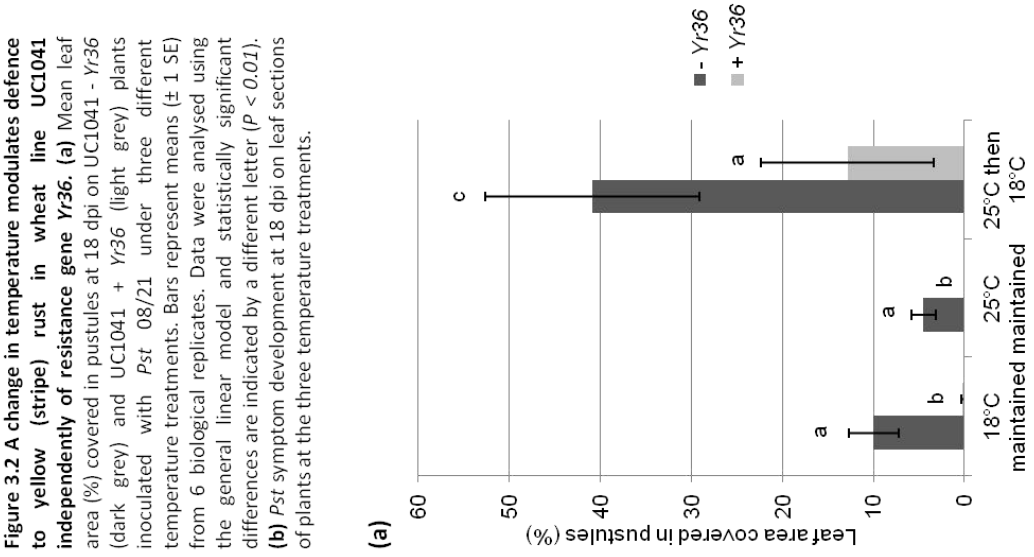




**Figure 3.2 A change in temperature modulates defence to yellow (stripe) rust in wheat line UC1041 independently of resistance gene *Yr36*.** **(a)** Mean leaf area (%) covered in pustules at 18 dpi on UC1041 - *Yr36* (dark grey) and UC1041 + *Yr36* (light grey) plants inoculated with *Pst* 08/21 under three different temperature treatments. Bars represent means (± 1 SE) from 6 biological replicates. Data were analysed using the general linear model and statistically significant differences are indicated by a different letter ($P < 0.01$). **(b)** *Pst* symptom development at 18 dpi on leaf sections of plants at the three temperature treatments.

**(a)**

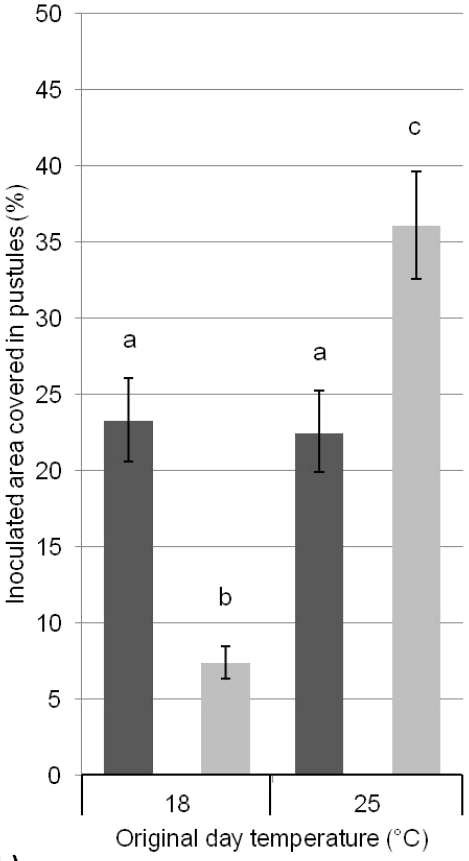
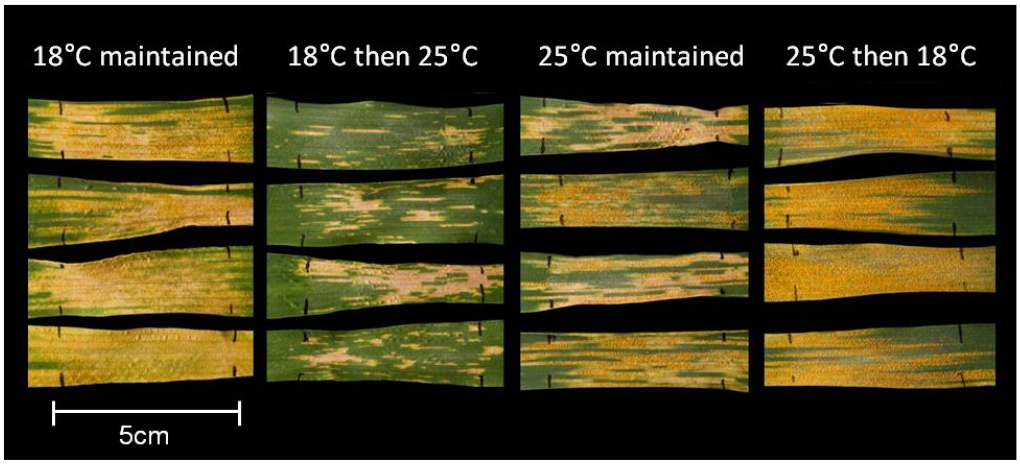



**Figure 3.3** A **temperature change in either direction influences resistance to *Pst* in UC1041 -*Yr36*. (a)** Mean inoculated area (%) covered in uredium pustules at 18 dpi on adult plants inoculated with *Pst* 08/21 and kept at 12°C/18°C or 12°C/25°C pre- and post-inoculation (dark grey) or transferred to the other temperature regime post-inoculation (light grey). Bars represent means predicted from the general linear model (± 1 SE) from three independent experiments. Different letters indicate a statistically significant difference ($P$ <0.01). **(b)** *Pst* symptom development at 18 dpi on plants in the four temperature regimes indicated by day temperature. Inoculated area is bounded by vertical lines.

Plants maintained at original temperature

Plants shifted to other temperature

**(b)**



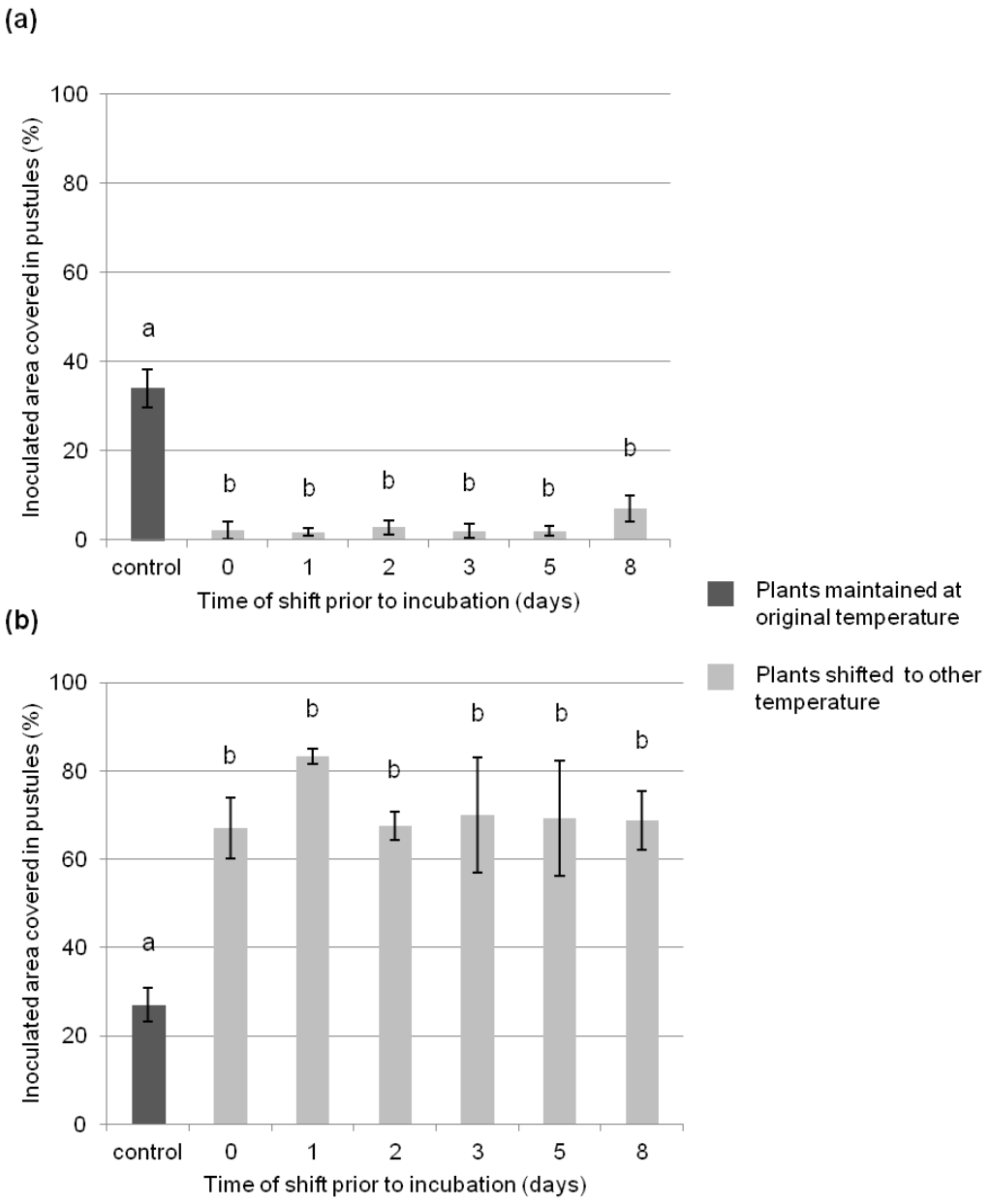
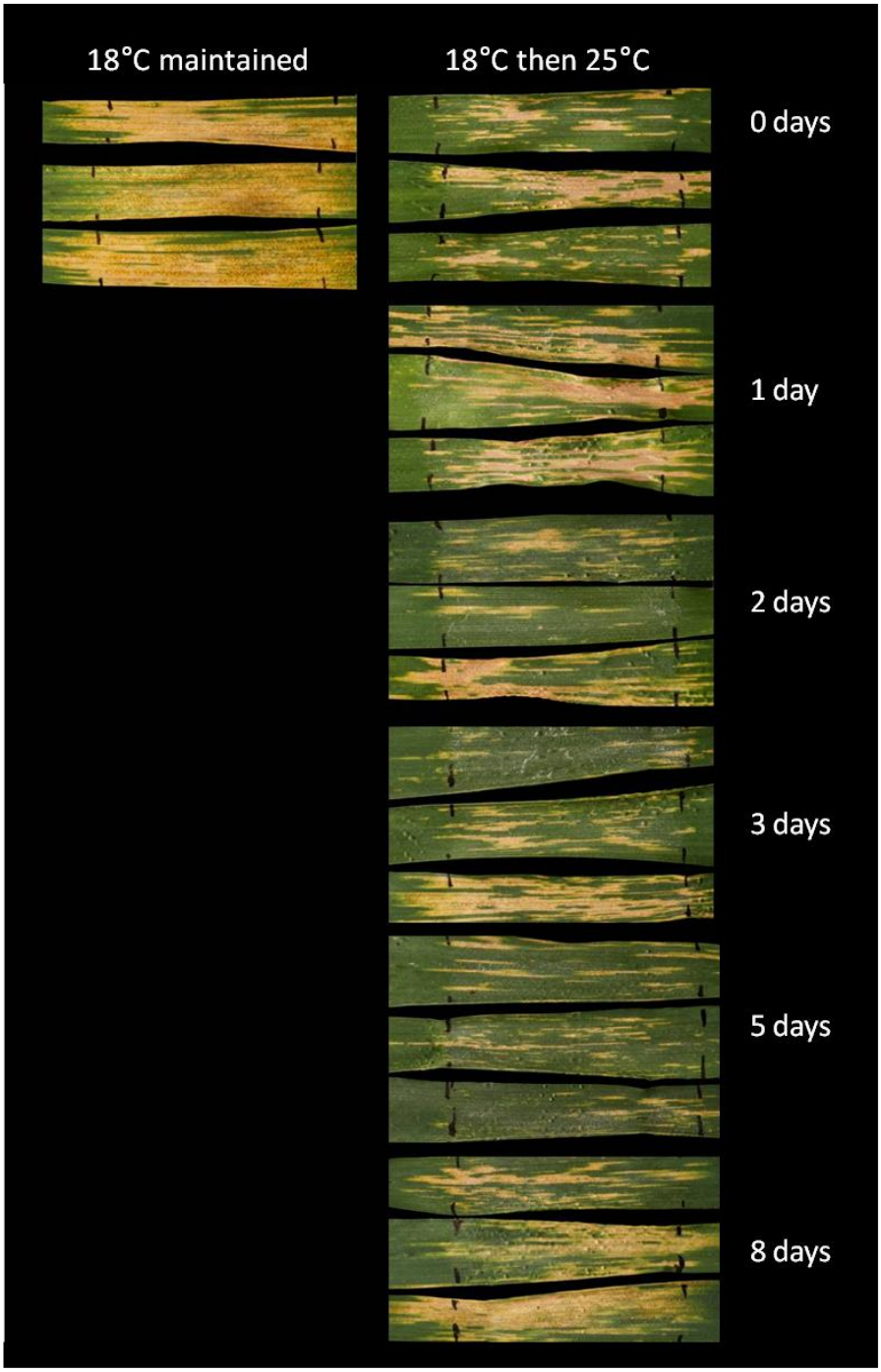
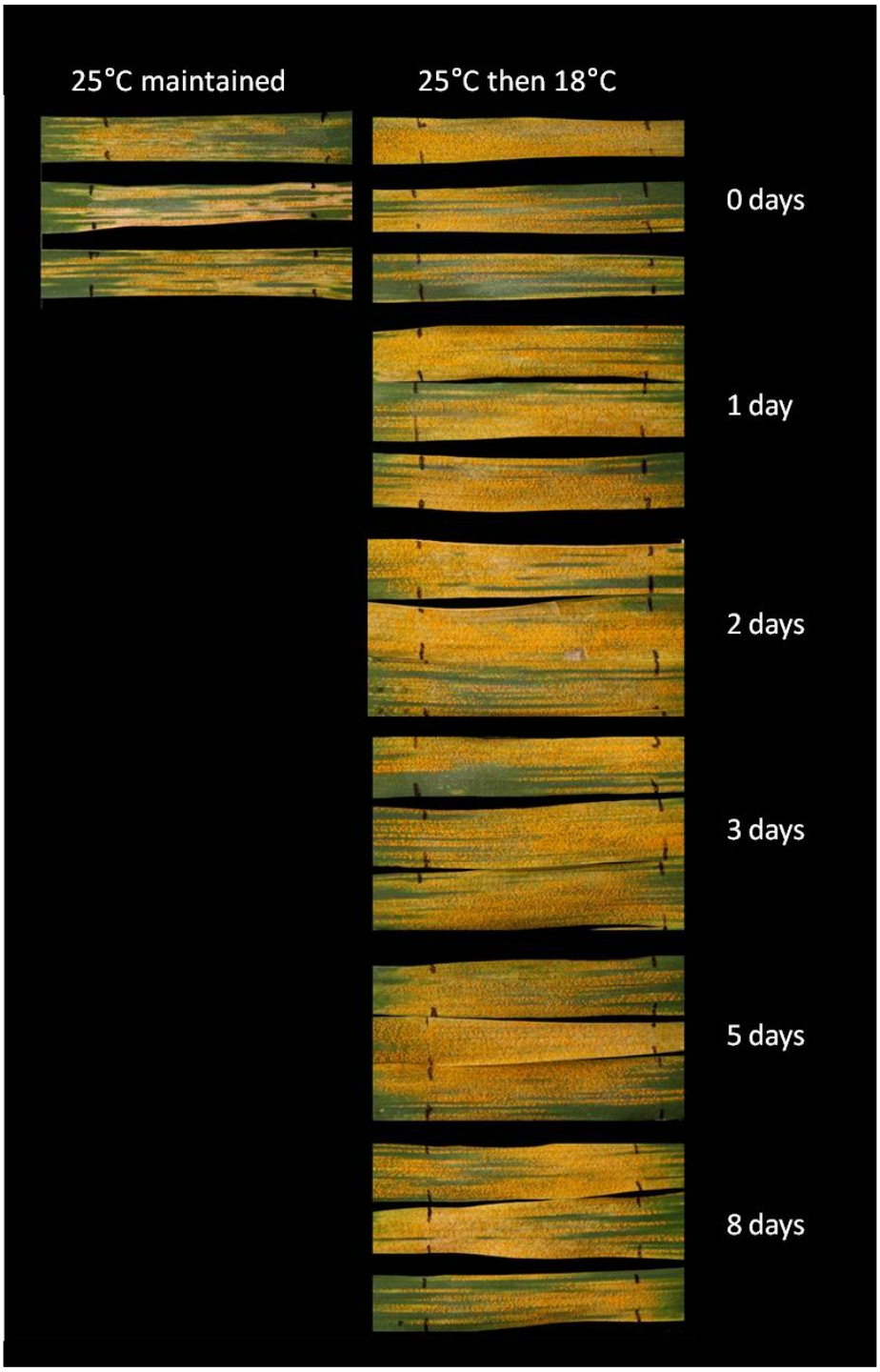



**Figure 3.4 Resistance to *Pst* is affected by temperature shifts prior to inoculation in wheat variety UC1041.** Mean inoculated area (%) covered in uredium pustules at 18 dpi on plants inoculated with *Pst* 08/21 and kept at 12°C/18°C or 12°C/25°C pre and post inoculation (dark grey) or transferred to the other temperature regime post-inoculation (light grey). Plants were originally grown at **(a)** 18°C and **(b)** at 25°C. Bars represent means (± 1 SE) from four biological replicates for which data were analysed with the general linear model. Different letters indicate a statistically significant difference ($P < 0.01$). **(c&d overleaf)** *Pst* symptom development at 18 dpi on leaf sections of plants at the four temperature treatments with various shift time points.

(c)


18°C maintained
18°C then 25°C
0 days
1 day
2 days
3 days
5 days
8 days

(d)


25°C maintained
25°C then 18°C
0 days
1 day
2 days
3 days
5 days
8 days

### 3.4.3 No obvious link is seen between the effect of an increase or decrease in day temperature on photosynthesis and the effect seen on resistance

Considering photosynthesis and defence are intrinsically linked (Göhre *et al*., 2012), the extent to which the temperature change could affect plant photosynthesis rate was investigated. Various gas exchange measurements were taken and *A*-$C_i$ curves determined for plants experiencing the four different temperature treatments in the absence of *Pst*. No significant differences in sub-stomatal $CO_2$ conductance at ambient $CO_2$ ($C_i$ at $C_a$ = 380) or $CO_2$ compensation point (Comp (Γ)) were observed between treatments at any of the time points (Table 3.1). At 1 day post treatment photosynthetic rate at ambient $CO_2$ in the presence ($A_i$) and absence ($A_a$) of stomatal limitation was significantly higher in plants that were grown in the 12/25°C regime then transferred to 12/18°C compared control treatments or the temperature change in the other direction. The *A*-$C_i$ curves suggest photosynthesis rates were higher in this treatment at both 4 and 8 days post temperature treatment although the difference was not significant at ambient $CO_2$ concentrations (Table 3.1, Figure 3.5). Other measurements calculated from the data showed significant differences between treatments but did not show a consistent pattern (Table 3.1).

### 3.4.4 A reduction in day temperature compromised *Yr39* mediated resistance in Alpowa

*Yr39* mediated resistance in Alpowa was also exposed to the temperature change at the time of inoculation with *Pst* to determine whether the resistance was affected. As in UC1041 +/- Yr36, no significant difference in percent pustule coverage was seen between plants maintained pre and post inoculation either 12/18°C or 12/25°C (Figure 3.6a,b). In addition there was no significant difference seen when plants experienced a temperature increase post-inoculation. However when plants experienced a reduction in temperature from 12/25°C to 12/18°C, plant pustule coverage significantly increased compared to all plants grown in all other temperature treatments ($P < 0.05$) except for plants maintained at 12/18°C which was almost significant ($P = 0.054$, Figure 3.6a,b).

**Table 3.1 Photosynthesis measurements of adult UC1041 plants under four different temperature treatments** Plants were kept at 12°C/18°C or 12°C/25°C or moved to the other regime and various measurements were taken using a Li-COR LI-6400. Measurements were performed at a RH of 70% and an irradiance of 1200 µmol $m^{-2}$ $s^{-1}$. Where $J_{max}$ is the maximum photosynthesis rate, $C_i$ at $C_a$ = 380 is substomatal $CO_2$ conductance at ambient $CO_2$ , $A_a$ is photosynthetic rate at ambient $CO_2$ in the absence of stomatal limitation, $A_i$ is photosynthetic rate at ambient $CO_2$ in the presence of stomatal limitation, I is stomatal limitation, CE is carboxylation efficiency of Rubisco and Comp (Γ) is $CO_2$ compensation point. Values are means (± S.E) from 3 plant individuals and different letters indicate a significant difference between treatments (*t-test value p-value < 0.05*).

| Time point | 1 day post shift | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Day temperature | 18°C maintained | | 18°C then 25°C | | 25°C maintained | | 25°C then 18°C | |
| $J_{max}$ (µmol $m^2$ $s^{-1}$) | 35.0 ± 5.0 | a | 37.6 ± 1.9 | a | 34.5 ± 1.0 | a | 41.6 ± 2.9 | a |
| $C_i$ at $C_a$ = 380 (µmol $mol^{-1}$) | 241.1 ± 46.2 | a | 272.9 ± 4.0 | a | 254.3 ± 16.5 | a | 243.1 ± 16.4 | a |
| $A_a$ (µmol $m^2$ $s^{-1}$) | 22.1 ± 3.0 | a | 23.4 ± 0.9 | a | 20.2 ± 0.4 | a | 26.1 ± 1.6 | b |
| $A_i$ (µmol $m^2$ $s^{-1}$) | 17.6 ± 1.1 | a | 19.7 ± 0.4 | a | 15.1 ± 0.6 | a | 20.8 ± 1.7 | b |
| I | 0.2 ± 0.1 | a | 0.2 ± 0.0 | a | 0.2 ± 0.0 | a | 0.2 ± 0.0 | a |
| CE (mmol $m^2$ $s^{-1}$) | 119.4 ± 24.6 | a | 111.0 ± 2.1 | a/b | 81.0 ± 4.6 | c | 119.4 ± 4.6 | a/b |
| Comp (Γ) (mmol $H_2O$ $m^2$ $s^{-1}$) | 48.9 ± 0.1 | a | 51.1 ± 3.8 | a | 52.5 ± 1.3 | a | 49.6 ± 1.1 | a |

| Time point | 4 day post shift | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Day temperature | 18°C maintained | | 18°C then 25°C | | 25°C maintained | | 25°C then 18°C | |
| $J_{max}$ (µmol $m^2$ $s^{-1}$) | 31.1 ± 1.6 | a/b | 37.0 ± 2.2 | a/b/c | 33.6 ± 3.2 | a/b | 44.2 ± 2.3 | c |
| $C_i$ at $C_a$ = 380 (µmol $mol^{-1}$) | 258.6 ± 4.8 | a | 229.2 ± 19.4 | a | 248.4 ± 7.2 | a | 189.0 ± 32.5 | a |
| $A_a$ (µmol $m^2$ $s^{-1}$) | 18.8 ± 1.5 | a | 21.7 ± 1.8 | a | 21.2 ± 2.0 | a | 23.2 ± 2.6 | a |
| $A_i$ (µmol $m^2$ $s^{-1}$) | 14.6 ± 1.6 | a | 16.0 ± 1.8 | a | 16.7 ± 2.0 | a | 12.1 ± 3.7 | a |
| I | 0.2 ± 0.0 | a | 0.3 ± 0.0 | a | 0.2 ± 0.0 | a | 0.5 ± 0.1 | b |
| CE (mmol $m^2$ $s^{-1}$) | 91.0 ± 0.9 | a | 101.4 ± 22.0 | a | 95.7 ± 8.0 | a | 76.5 ± 19.5 | a |
| Comp (Γ) (mmol $H_2O$ $m^2$ $s^{-1}$) | 50.2 ± 1.7 | a | 46.6 ± 1.9 | a | 45.9 ± 2.9 | a | 56.1 ± 6.5 | a |

| Time point | 8 day post shift | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Day temperature | 18°C maintained | | 18°C then 25°C | | 25°C maintained | | 25°C then 18°C | |
| $J_{max}$ (µmol $m^2$ $s^{-1}$) | 31.1 ± 1.0 | a/b | 33.8 ± 1.6 | b | 30.2 ± 0.6 | c | 35.8 ± 1.3 | a/b/c |
| $C_i$ at $C_a$ = 380 (µmol $mol^{-1}$) | 237.1 ± 4.7 | a | 243.5 ± 8.6 | a | 255.6 ± 1.0 | a | 241.1 ± 11.6 | a |
| $A_a$ (µmol $m^2$ $s^{-1}$) | 20.2 ± 1.0 | a/b/c | 20.8 ± 0.6 | a/b | 18.8 ± 0.5 | c | 22.0 ± 0.4 | a/b |
| $A_i$ (µmol $m^2$ $s^{-1}$) | 15.3 ± 1.2 | a | 15.6 ± 0.9 | a | 14.6 ± 0.6 | a | 16.2 ± 1.0 | a |
| I | 0.2 ± 0.0 | a | 0.3 ± 0.0 | a | 0.2 ± 0.0 | a | 0.3 ± 0.0 | a |
| CE (mmol $m^2$ $s^{-1}$) | 86.2 ± 5.8 | a/b/c | 75.9 ± 3.4 | a/b | 77.3 ± 1.3 | a/b | 100.4 ± 9.9 | c |
| Comp (Γ) (mmol $H_2O$ $m^2$ $s^{-1}$) | 43.2 ± 1.6 | a | 46.9 ± 3.3 | a | 42.4 ± 0.5 | a | 42.3 ± 0.8 | a |

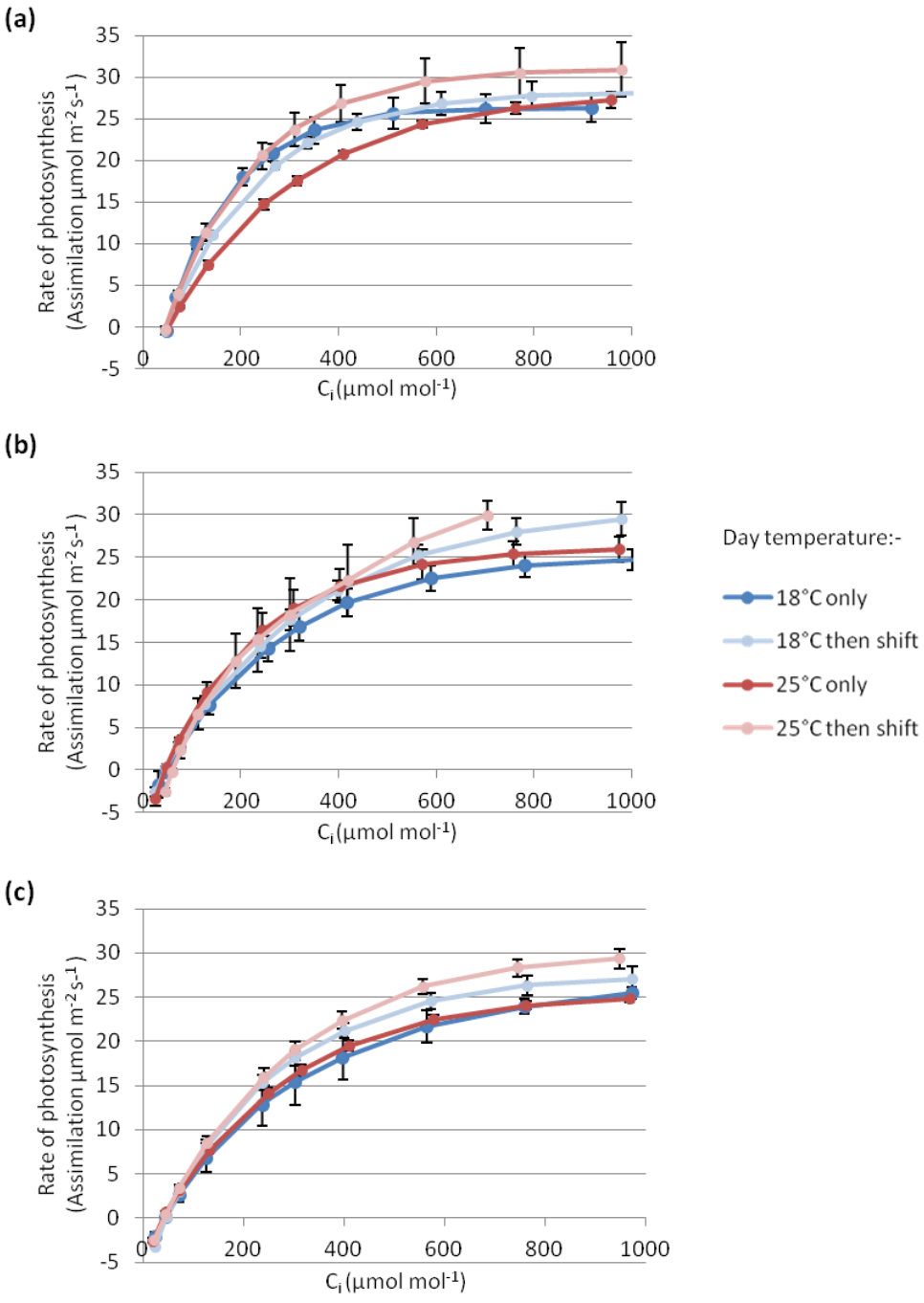



**Figure 3.5 *A*-$C_i$ curves showing photosynthesis rates in plants experiencing one of four different temperature treatments.** Plants were grown to flag leaf stage at 12/18°C or 12/25°C and either left in the same temperature regime or transferred to the other regime. Gas exchange measurements were taken with a Li-COR LI-6400 at **(a)** 1 day, **(b)** 4 days and **(c)** 8 days post treatment and the data were used to generate *A*-$C_i$ curves. Data point show mean photosynthesis rate (± 1 SE) at a range of $CO_2$ concentrations.

(a)

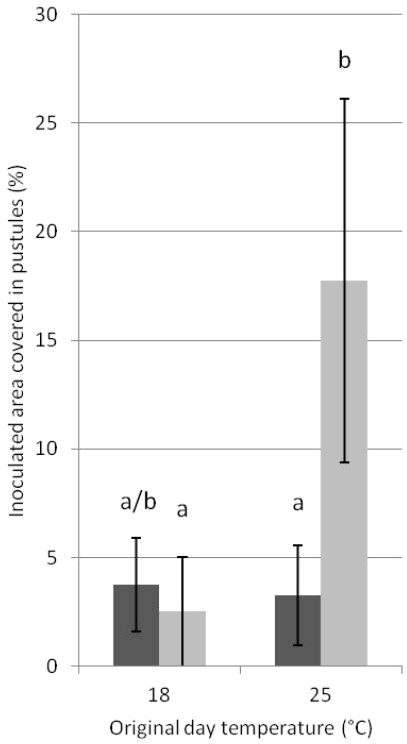
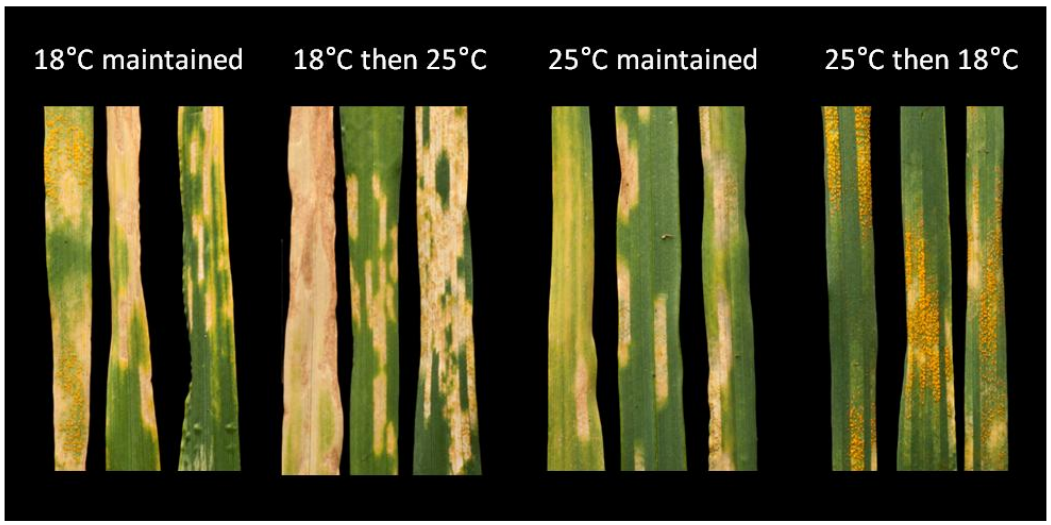



**Figure 3.6 A temperature change compromises *Yr39* mediated resistance in Alpowa (a)** Mean inoculated area (%) covered in uredium pustules at 18 dpi on plants inoculated with *Pst* 08/21 at growth stage 28 (Zadok) and kept at 12°C/18°C or 12°C/25°C pre and post inoculation (dark grey) or transferred to the other temperature regime post-inoculation (light grey). Bars represent means (± 1 SE) from four plant individuals. Different letters indicate a statistically significant difference ($P$ <0.05) using general linear regression. **(b)** *Pst* symptom development at 18 dpi on plants in the four temperature regimes indicated by day temperature.

(b)



### 3.4.5 An increase in temperature can affect *Pst* resistance in seedling UC1041 -*Yr36* but the phenotype is inconsistent

No resistance was observed in seedling UC1041 -*Yr36* plants infected with *Pst* 08/21, resulting in higher levels of pustules compared to adult plants. As in adult UC1041 plants, there was no significant difference in disease levels between plants which were maintained at either 12/18°C or 12/25°C pre and post-inoculation (Figure 3.7a,b). There was also no significant difference in pustule levels between plants maintained at 12/25°C and plants transferred from 12/25°C to 12/18°C after *Pst* inoculation. As in adult plants, enhanced resistance was occasionally observed when seedlings were transferred from 12/18°C to 12/25°C ($P < 0.001$, Figure 3.7a,b), although the phenotype was not always observed. Further investigations were performed on UC1041 seedlings rather than adults, to enable a greater number of experiments to be performed.

### 3.4.6 A reduction in day temperature affects later stages of *Pst* colonisation UC1041 seedlings

When enhanced resistance resulting from the transfer to a higher temperature was seen in UC1041 seedlings, colonisation and progression of *Pst* was observed microscopically. There was no significant difference in the percentage of germinated uredospores between all four temperature treatments at both 1 dpi (Figure 3.8a) and 3 dpi (Figure 3.8c). Similarly, there were no significant differences in percentage of germinated uredospores forming SSVs between the four temperature regimes at either 1 dpi (Figure 3.8b) or 3 dpi (Figure 3.8d). At 6 dpi, plants grown at 12/25°C then transferred to 12/18°C post-inoculation had significantly smaller internal fungal structures ($P < 0.001$) compared to all other treatments (Figure 3.9a). By 8 dpi, *Pst* sub-cellular hyphal colonisation was less in plants originally grown at 12/18°C post-inoculation compared to those grown at 12/25°C, regardless of the temperature change ($P < 0.001$, Figure 3.9b,c). Plants grown at 12/18°C and then transferred to the higher temperature regime after *Pst* inoculation showed significantly less hyphal colonisation than plants maintained at 12/18°C ($P < 0.001$). Hyphal growth in plants at 12/25°C pre and post *Pst* inoculation did not differ significantly from plants transferred from 12/25°C to 12/18°C after inoculation (Figure 3.9b,c).

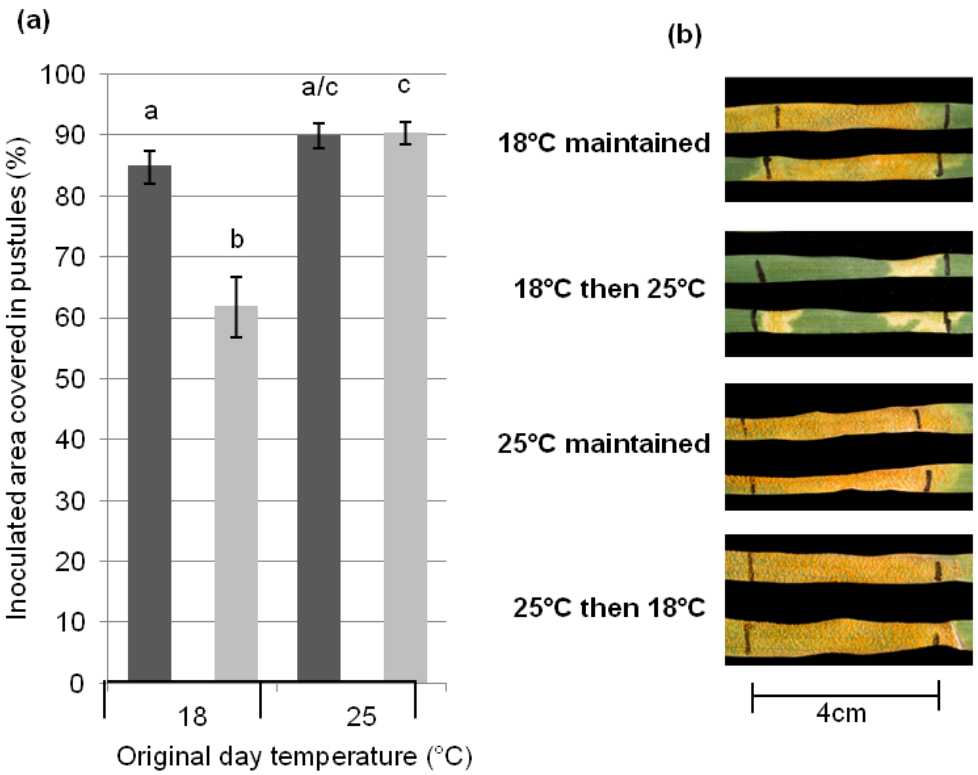



**Figure 3.7 An increase in day temperature can enhance resistance in UC1041 seedlings on some occasions. (a)** Mean inoculated area covered in pustules (%) at 18 dpi on UC1041 seedlings inoculated with *Pst* 08/21 and kept at 12°C/18°C or 12°C/25°C pre and post inoculation (dark grey) or transferred to the other temperature regime post-inoculation (light grey). Inoculated area is bounded by vertical lines. Bars represent means predicted from the general linear model (± 1 SE) from at least three independent experiments. Different letters indicate a statistically significant difference ($P < 0.01$). **(b)** *Pst* symptom development at 18 dpi on leaf sections of plants at the four temperature treatments.

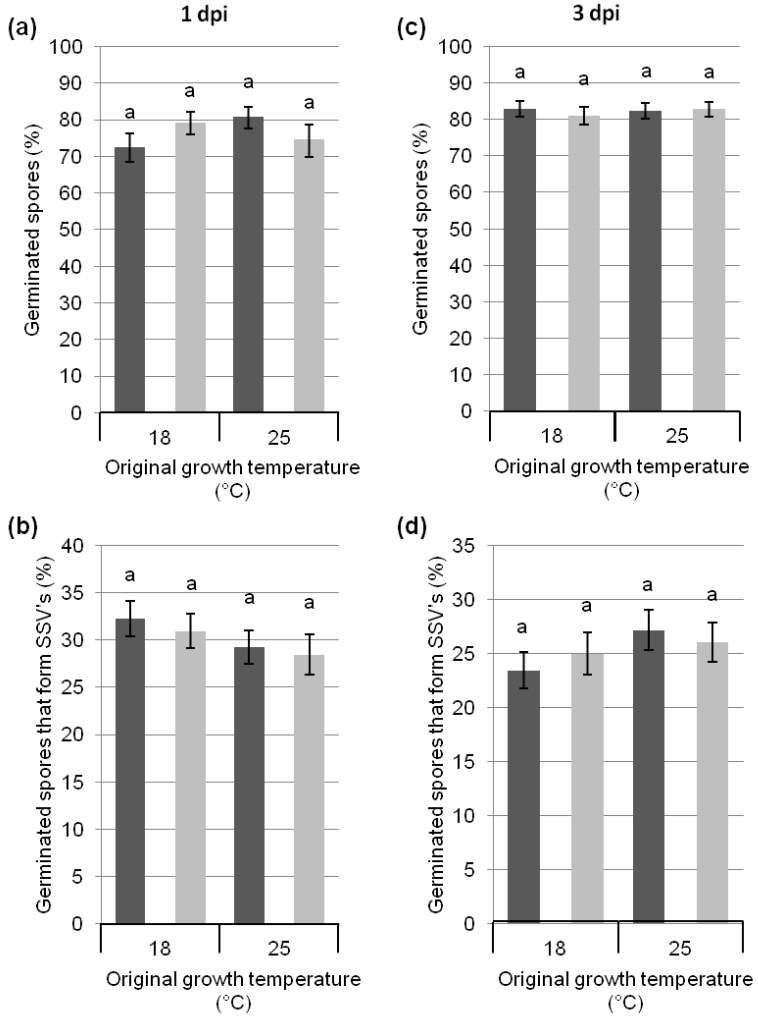



**Figure 3.8** The **temperature shift affects later stages of pathogen development in wheat variety UC1041.** UC1041 seedlings were inoculated with *Pst* and kept at the same temperature regime of 12°C/18°C or 12°C/25°C pre and post inoculation (dark grey) or transferred to the other regime post inoculation (light grey) then analysed microscopically. **(a)** *Pst* spore germination levels (%) at 24 hrs post inoculation. **(b)** Germinated spores forming SSVs at 24 hrs post inoculation (%). **(c)** *Pst* spore germination (%) in inoculated region at 72 hrs post inoculation. **(d)** Germinated spores able to form SSVs (%) at 72 hrs post inoculation. Bars represent means predicted from the general linear model (± 1 SE) from two independent experiments. Different letters indicate a statistically significant difference ($P < 0.05$).

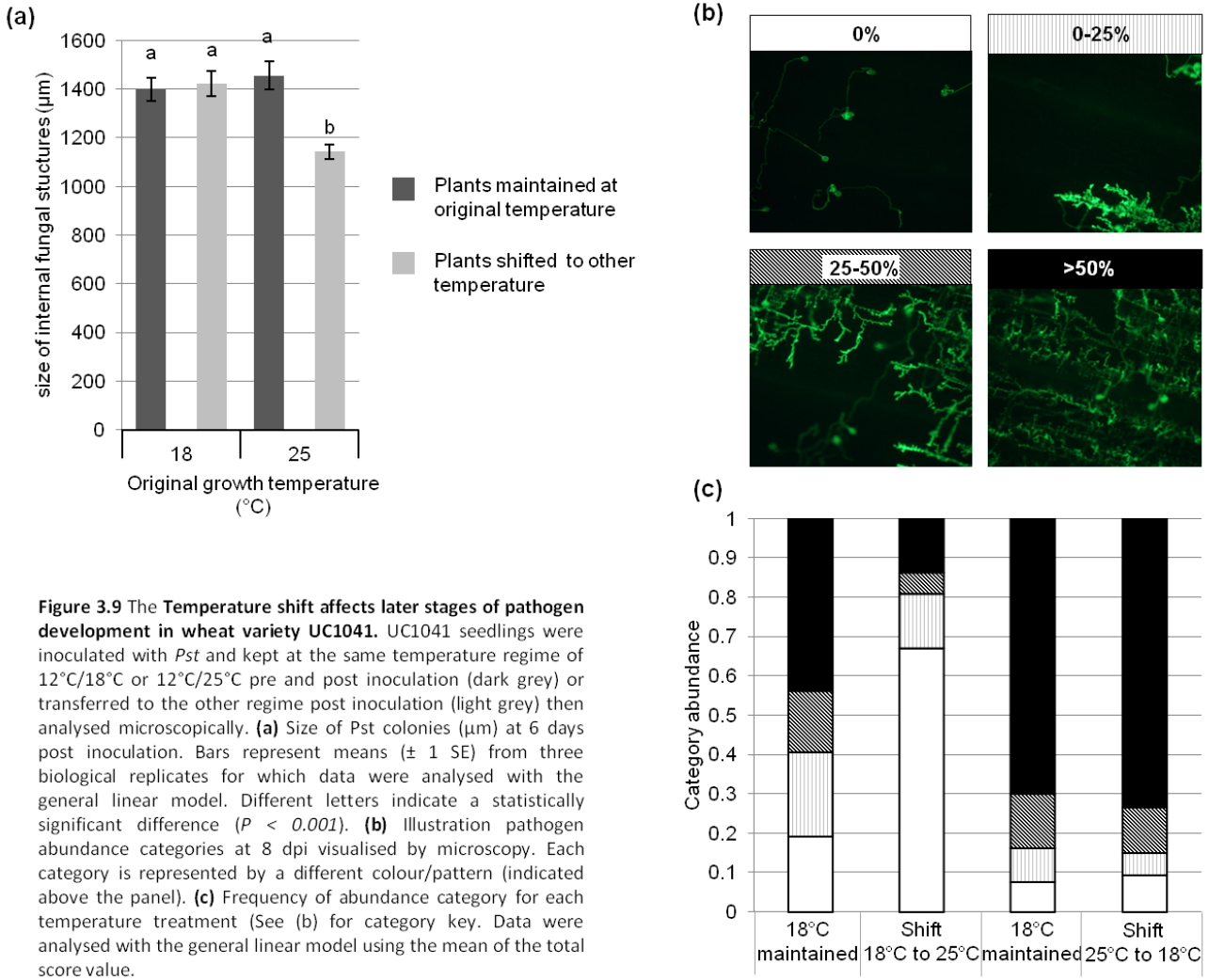



**Figure 3.9** The **Temperature shift affects later stages of pathogen development in wheat variety UC1041.** UC1041 seedlings were inoculated with *Pst* and kept at the same temperature regime of 12°C/18°C or 12°C/25°C pre and post inoculation (dark grey) or transferred to the other regime post inoculation (light grey) then analysed microscopically. **(a)** Size of Pst colonies (µm) at 6 days post inoculation. Bars represent means (± 1 SE) from three biological replicates for which data were analysed with the general linear model. Different letters indicate a statistically significant difference ($P < 0.001$). **(b)** Illustration pathogen abundance categories at 8 dpi visualised by microscopy. Each category is represented by a different colour/pattern (indicated above the panel). **(c)** Frequency of abundance category for each temperature treatment (See (b) for category key. Data were analysed with the general linear model using the mean of the total score value.

### 3.4.7 Temperature shifts do not affect host resistance to *Pst* in other wheat cultivars

To determine whether the temperature-shift induced resistance to *Pst* was observed in other wheat cultivars, the same experimental procedures were used on seedlings of selected UK elite varieties. Resistance to *Pst* in Shamrock displayed a similar phenotype to UC1041, with plants that were transferred from lower to higher temperatures displaying a significant reduction in pustule levels ($P < 0.05$, Figure 3.10a). However resistance in Solstice was not affected by transferring between temperature regimes in either direction as disease levels were not significantly different between all four treatments (Figure 3.10b). As in seedling UC1041 plants, resistance enhancement in Shamrock when plants were transferred from 12/18°C to 12/25°C was not always observed (1 in 4 experiments did not see any effect of increased temperature on resistance). The same experiment was carried out separately on Sappo plants. Like Solstice, defence in this cultivar appeared to be similarly uninfluenced by temperature shifts, since pustule levels in Sappo were not significantly different between temperature treatments (Figure 3.11) However, the experiment on Sappo was not run in conjunction with Solstice and Shamrock so it is not possible to make a direct comparison.

## 3.5 Discussion

Our investigations show that *Yr36* can prevent uredia formation of *Pst* isolate 08/21 at temperatures as low as 18°C, rather than the higher than 20°C temperature limit previously reported (Fu *et al.*,2009). The result here provides an explanation for the earlier report since, in those investigations, UC1041 *+Yr36* plants were exposed to similar decreases in temperature at the time of inoculation. In addition, we discovered that the UC1041 genetic background responds to changes in temperature independently of *Yr36,* affecting growth of *Pst.* Our results suggest that *Yr36*-mediated resistance may be affected by a previously-uncharacterised background response to temperature changes present in UC1041. In the field, *Yr36* conveys a QDR phenotype on adult plants in Mediterranean environments (Uauy *et al.,*2005). Based on the results presented here, we hypothesise that the QDR observed in the field is due to exposure of the UC1041 + *Yr36* line to frequent temperature changes. Furthermore, our results also suggest that *Yr36*-mediated resistance should be effective in the field under relatively cool temperate conditions.

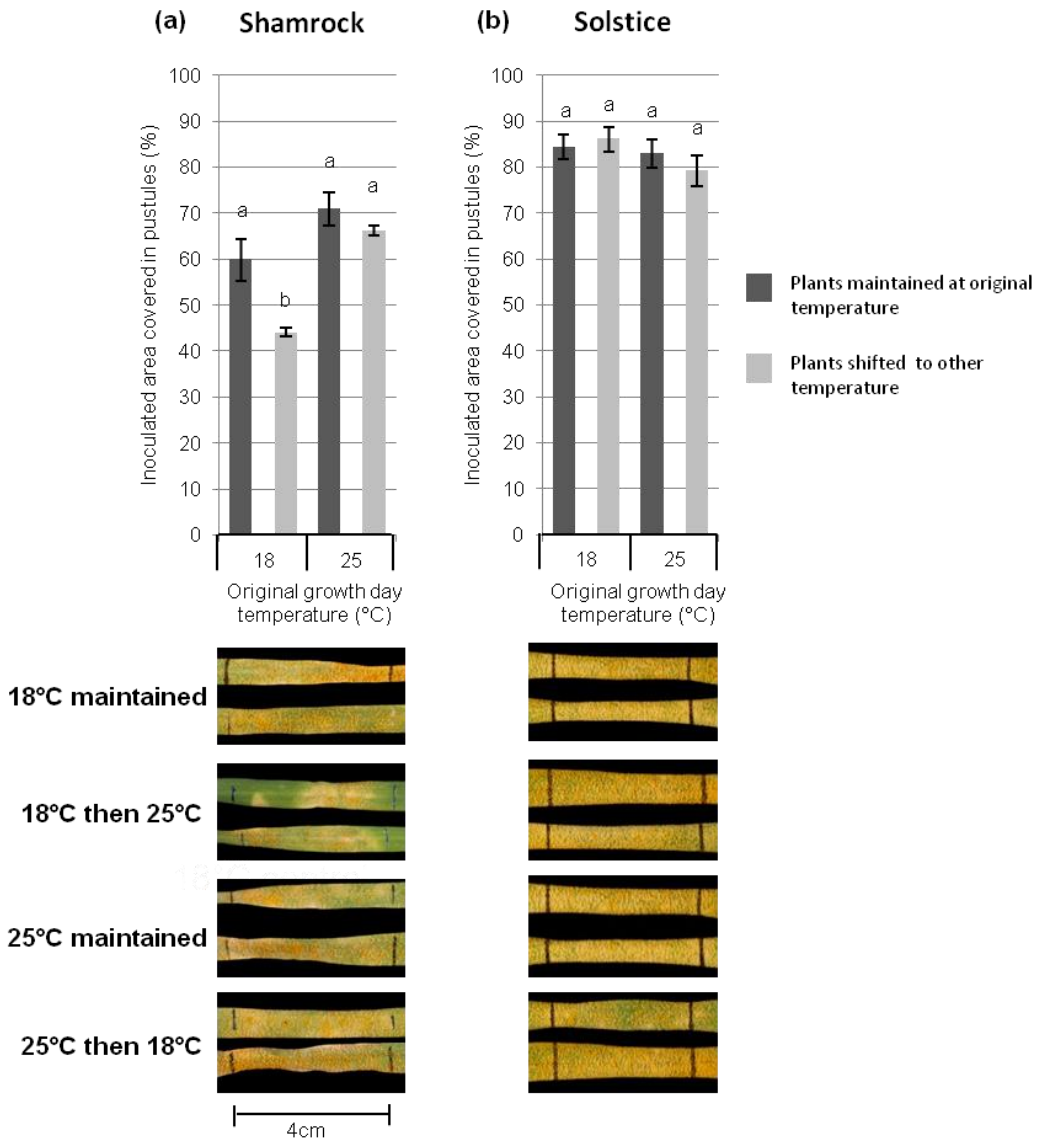



**Figure 3.10 Temperature shifts do not affect resistance to *Pst* in other wheat cultivars.** Percentage of inoculated leaf area covered in uredium pustules at 18 dpi on two cultivars of young wheat plants inoculated with *Pst* and kept at the same temperature regime of 12°C/18°C or 12°C/25°C pre and post inoculation (dark grey) or transferred to the other regime post inoculation (light grey). Columns show graphical and visual representations from cultivars: **(a)** Shamrock, and **(b)** Solstice. Inoculated area is bounded by vertical lines. Bars represent means predicted from the general linear regression model (± 1 SE) from at least two independent experiments where cultivars Shamrock and Solstice were inoculated at the same time. Different letters indicate a statistically significant difference ($P < 0.05$).

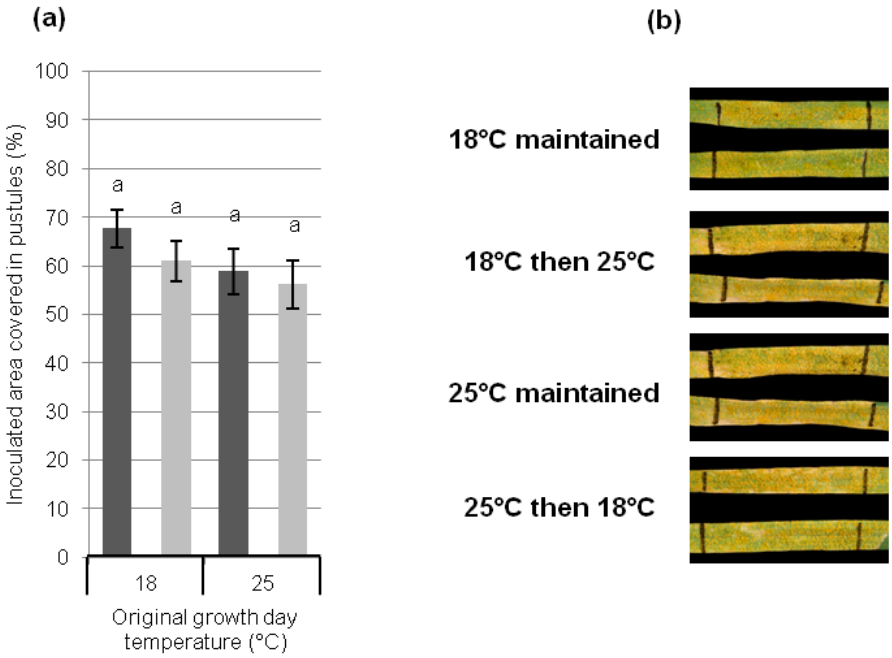



**Figure 3.11 Temperature shifts may not affect resistance to *Pst* in cultivar Sappo.** **(a)** Percentage of inoculated leaf area covered in uredium pustules at 18 dpi on Sappo seedlings inoculated with *Pst* and kept at the same temperature regime of 12°C/18°C or 12°C/25°C pre and post inoculation (dark grey) or transferred to the other regime post inoculation (light grey). Bars represent means predicted from the general linear regression model (± 1 SE) from at least two independent experiments where Sappo was inoculated independently of other cultivars. Different letters indicate a statistically significant difference ($P < 0.05$). **(b)** *Pst* symptom development at 18 dpi on plants in the four temperature regimes. Inoculated area is bounded by vertical lines.

Interestingly, *Yr39*-mediated resistance in cultivar Alpowa is also compromised by a temperature reduction. Since NIL's are not available for *Yr39*, it is not possible to determine whether temperature sensitivity conferred directly by *Yr39* or is present in the Alpowa background as in UC1041. It is also difficult to compare with results seen in UC1041 since Alpowa was inoculated at a different developmental stage from UC1041 plants. Several studies characterising temperature-sensitive resistance genes are performed with different pre- and post-inoculation conditions, but the effect of temperature changes is not considered. As with UC1041, it is possible that genes responding to changes in temperature and affecting resistance may be present in other cultivars. The extent of resistance may therefore be affected by temperature fluctuations rather than requiring exposure to a temperature threshold (Broers and Wallenburg, 1989; Dyck and Johnson, 1983; Ramage and Sutherland, 1995). For example Broers and Wallenburg (1989) observed that a decrease in temperature increases *Lr34/Yr18* mediated resistance. However, control plants were not grown at a constant lower temperature before inoculation, so it is not possible to assess whether it was the decrease or changes in temperature that was responsible for the enhanced resistance. Pretorius *et al* (1994) later point out that the study by Broers and Wallenburg (1989) does not exclude background effects from the cultivars in which the *Lr34/Yr18* gene resides. The results with *Yr36* and *Yr39* in this study highlight the importance of controls in pathology studies when characterising temperature-sensitive genes.

Studies with UC1041 seedlings revealed that a temperature increase can enhance resistance to *Pst* at an early stage of plant development. However, the results are less consistent than those observed in adult plants. Also in seedlings, a decrease in temperature does not reduce resistance to *Pst* as seen in adult plants of UC1041 (40% infection), but this could be due to the higher levels of *Pst* infection seen on UC1041 seedlings (90% infection). Differences between adult plants and seedlings could be caused by *Pst* inoculum levels, or reflect physiological differences influencing defence, with adult plants being more responsive to temperature changes than seedlings (Basnet *et al.*, 2013). As in UC1041, the enhanced resistance observed in Shamrock was not seen in all seedling experiments. When the phenotype was not observed, uredia abundance was higher in both cultivars compared to experiments where a temperature effect was observed. This suggests that high levels of *Pst* inoculum and subsequent heavy infection loads may mask the effect of the temperature change.

The evidence indicates that *Pst* is able to germinate and penetrate the plant successfully, regardless of the temperature change, indicating that resistance conferred by initial recognition of the pathogen is not affected by a change in temperature. The phenotype observed when UC1041 and Shamrock plants were transferred from 12/18°C to 12/25°C resembles that of 'late' or 'slow' rusting resistance. Slow rusting is a type of QDR normally associated with phenotypes such as increased latency period, or decreased infection frequency and uredium size (Herrera-Foessel *et al*., 2012; Lee and Shaner 1984; Rubiales and Niks, 1995; Shah *et al*., 2010; William *et al*., 2006). Disease in slow-rusting resistance is not generally seen early in the infection cycle, but occurs later in the season. Some aspects of the phenotype are comparable to HTAP yellow rust resistance which is a type of QDR generally effective after stem elongation and when day temperatures are 25°C–30°C (Coram *et al*., 2008). However, the enhanced resistance that was observed was induced by an increase in temperature rather than prolonged exposure to 25°C, and can also occur in seedlings. The results suggest that temperature changes, rather than exposure to a threshold temperature, could be influencing some QDR mechanisms.

The basis for the observations in this chapter could be due to the modulation of hormonal and defence pathways by temperature changes. Plants adapt continuously to changing environments and balance resources between growth and defence to achieve maximum productivity (Koga *et al*., 2004a; Mosher *et al*., 2010). When moved to a different temperature, plants need to adapt to the new conditions. Studies with the model plant *Arabidopsis thaliana* suggest a general trade-off mechanism in plants whereby hormone-mediated growth may antagonise immune responses (Albrecht *et al*., 2012; Anderson *et al*., 2004). Temperature changes could alter the hormonal balance in favour of either defence or growth. Results showed that plants that experienced a reduction in day temperature have higher photosynthesis rates which could suggest a balance in favour of growth over defence. However evidence suggests that this isn't the cause for reduced resistance since plants experiencing an increase in temperature do not have a reduced rate of photosynthesis. A more likely explanation is that the temperature change could have an indirect effect on later stage basal defence mechanisms in UC1041, thereby reducing pathogen abundance. For example the temperature change could trigger (i) reorganisation of energy supplies resulting in reduced nutrient availability to the biotrophic rust fungus (Grof *et al*., 2010; Viola & Davies, 1994) or (ii) lead to production and/or accumulation of pathogen-deterring metabolites (Berger *et al*., 2007; Hu *et al*., 2013; Mazid *et al*., 2011).

Although QDR mechanisms are largely unknown, *Lr34* and *Yr36* do not fall into the general NBS-LRR class of R genes so it is reasonable to hypothesise that these are not generally involved in pathogen recognition (Thordal-Christensen, 2003). Some QDR genes may have other functions that indirectly affect pathogen development when exposed to changes in temperature.

Our results show that a change in temperature up to 8 days before inoculation affected defence against *Pst* in UC1041 adult plants. Thus, pre-exposure to a different temperature regime affects subsequent defence, which suggests an adaptive response. We have no evidence that the temperature change is resulting in a stress response, but the lasting effect of increased or decreased resistance is comparable to priming whereby previous exposure of plants to stress enables a faster response to subsequent stresses (Conrath *et al*., 2006). Ambient temperature changes have been shown to cause adaptive change through epigenetic modification of DNA activity by methylation (Kumar and Wigge, 2010). Correspondingly in wheat, an ambient temperature change could also epigenetically prime plants, affecting later stages of defence in UC1041.

Seedlings of Shamrock demonstrated a similar resistance phenotype to UC1041 when plants were transferred to a higher temperature post-inoculation. However, resistance in Solstice seedlings was not affected by the temperature change in either direction, indicating that the response varies between cultivars. Observations from Sappo plants indicate that Solstice may not be unique in this trait, although it is difficult to say for certain considering the experiment was not run simultaneously on the two varieties. Results of Park *et al* (1992) support observations of differences in resistance sensitivity between wheat cultivars when challenged with *Pst* at different pre and post inoculation temperature regimes. The authors attributed enhanced resistance at higher temperatures to factors that control adult plant resistance (APR) because this was present in the cultivars used in their study. The presence of additional APR genes cannot be discounted as both UC1041 and Alpowa do have varying levels if APR. Confirmation of APR in Solstice would further determine whether this hypothesis is valid. If the factors that control APR resistance do contribute to temperature sensitivity in seedlings, then this sensitivity is not lost at adult growth stage in UC1041. Resistance that shows resilience against temperature fluctuations may also occur against other wheat pathogens. For example, a significant difference in response to changing temperatures was observed between wheat cultivars in defence

against *Blumeria graminis* f. sp *tritici*, which causes powdery mildew, although this was attributed to R genes showing different levels of temperature sensitivity (Ge *et al*., 1998).

The results in this chapter suggest genetic diversity exists for resistance that is resilient to temperature changes in yellow rust. However the experimental design based on work by Fu *et al* (2009) is not ideal to explore this hypothesis, due to over complicating factors such as differences in average temperature between regimes. *Pst* is also not an ideal pathogen to work with considering it is an obligate biotroph and cannot be grown independently of plant tissue to see how the temperature treatments affect *in vitro* growth of the fungus. Further experiments are required to determine whether this phenotype is (i) seen in other plant–pathogen interactions (ii) consistent in adults of various cultivars and (iii) a trait that can be genetically mapped. Resilience to changes in temperature could be a valuable trait in breeding wheat cultivars with more environmentally stable resistance to *Pst* and other pathogens, especially if its effects can over-ride QDR genes. It would also be valuable to know whether the temperature changes can diminish complete resistance conferred by R genes as this could contribute to reduced durability. Breakdown of R genes that confer resistance to specific isolates of *Pst* are generally associated with weather conditions that are favourable to spread of the pathogen due to increased generations cycles causing increased mutation rate. However there is no knowledge of whether the environmental impact on host defence contributes to this process. In addition, the results indicate that decreases in temperature could make particular cultivars more vulnerable to pathogen infection in the field, if temperature drops increase susceptibility (Figure 3.12). Conversely it is naïve to argue that plants that have never experienced a change in daily regime temperature are comparable to plants grown in a natural environment where they are exposed to frequent temperature changes and multiple stresses. A valuable research study could be to investigate whether warmer periods of weather followed by cooler periods historically results in yellow rust outbreaks and whether this could be due to compromised resistance. Temperature changes occur frequently in the natural environment and are predicted to become more common as the climate changes (Asseng *et al*., 2011). Further research has the potential to identify vulnerable periods where epidemics are more likely to occur and this information could be used to inform fungicide application through the use of modelling.

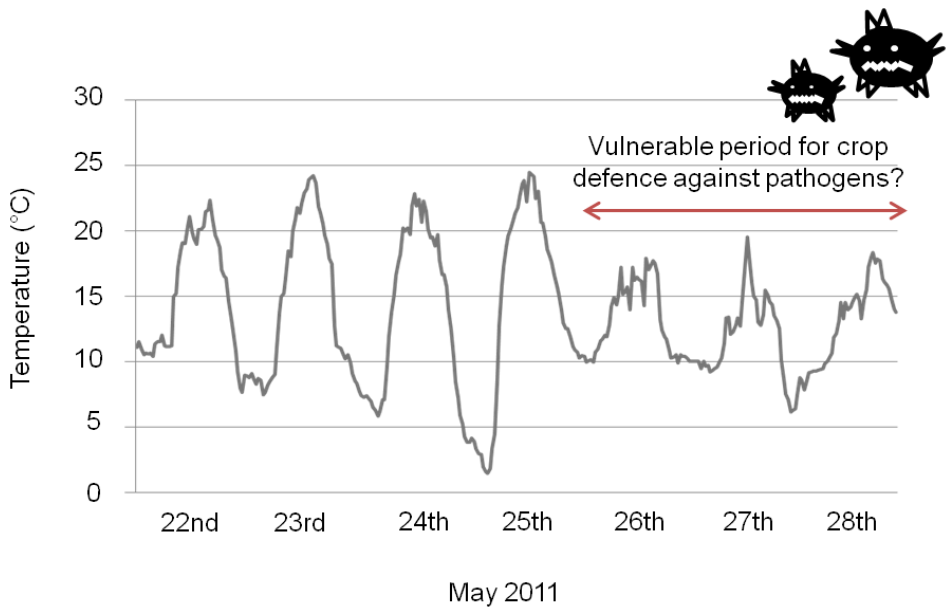



**Figure 3.12 Drops in temperature could make crop defence to *Pst* more vulnerable.** Field temperature measurements illustrating changing conditions similar to those used in the experiment. Temperature recordings were taken every 30 mins in 2011 at the John Innes field trial site using SL54TH® data loggers (Signatrol Ltd, Tewkesbury, UK).

In conclusion, *Yr36* has potential to be useful in UK agriculture considering that it does not need prolonged exposure to high temperatures in order to function. However, the results show that *Yr36*-mediated resistance could be compromised by temperature decreases, although further investigation is needed to ascertain how consistent this is. More importantly, findings reveal a possible novel trait for resistance stability under varying temperatures. Consistent crop performance and reliable disease resistance are important targets in plant breeding. Understanding effects of temperature changes on plant defence will be essential for developing crops that are more resilient to the potential impacts of climate change.

# Chapter 4: Resistance to different pathogen types under constant and varying temperatures

## 4.1 Aim:

This primary aim of the work described here was to determine whether there are differences in the disease resistance response of wheat cultivars under constant and varying temperatures.

## 4.2 Introduction

Chapter 3 described how the influence of a change in temperature on resistance to *Pst* may vary between cultivars. From the results presented, it is reasonable to hypothesise that defence mechanisms in some wheat cultivars are more susceptible to changes in temperature than in other cultivars. It was also proposed that temperature changes affect defence generally and may not be specific to *Pst*. To investigate this further, additional cultivars need to be tested against different pathogens. However, the temperatures used in chapter 3 are not conducive to some pathogens, which prevented investigation into general defence mechanisms beyond those that might be specific to *Pst*. To progress the study, a simplified temperature regime was developed that enabled additional cultivars and pathogens to be investigated and in addition to *Pst*, the pathogens *Blumeria graminis* f. sp *tritici* (*Bgt*), *Fusarium culmorum* and *Pseudomonas syringae* pv. *oryzae* were included in the study. *Bgt* causes wheat powdery mildew disease and is a biotrophic pathogen like *Pst* whereas *F. culmorum* is necrotrophic. Procedures for the inoculation of cereal leaves by *F. culmorum* have been developed (Chen *et al*., 2009). The study therefore enables comparison of the effects of temperature changes on resistance against necrotrophic and biotrophic pathogens. *P. syringae* is routinely used in disease resistance assays in Arabidopsis, and has the advantage of being readily cultivated and quantified, so could potentially be used for screening many wheat cultivars. Previous work identified *Por*36_1 strain as compatible with wheat (Schoonbeek *et al*., *in press*).

As discussed in chapter 3, there has been much investigation into how different average temperatures can affect resistance (Whitham *et al*., 1996; Xiao *et al*., 2003; Yang and Hua, 2004) and limited exploration of the effects of a change in temperature pre- and post-

inoculation (Park *et al*., 1992; Ramage and Sutherland 1995). The time of exposure of a plant to a particular temperature during a day/night cycle is known as a thermoperiod. The ability of a plant to cope with a pathogen under varying thermoperiods opposed to constant temperatures has not been explored, and is the subject of this chapter.

The possible involvement of DELLA-mediated responses to temperature changes are discussed in this chapter. DELLA proteins are core components of the Gibberellic Acid (GA) pathway and act to repress GA mediated growth by interacting with the soluble GA receptor, GIBBERELLIN INSENSITIVE DWARF1, GID1 and the F-box protein SLY1/GID2 as shown in *Arabidopsis* (Ueguchi-Tanaka *et al*., 2005). When GA accumulates, DELLA proteins are degraded, thus releasing the growth restraint (Bonetta *et al*., 2005). Studies in *Arabidopsis* (Navarro *et al*., 2008), and more recently in wheat (Saville *et al*., 2012) have shown that DELLA proteins are implemented in increased susceptibility to biotrophic pathogens and reduced susceptibility to necrotrophs.

Heat shock proteins are responsible for protein folding, translocation and degradation under normal conditions and in addition, can assist in protein folding under stress conditions (Al-whaibi, 2011). Kumar and Wigge (2010) observed that *HSP70* transcript was expressed at a level proportional to the ambient temperature between 12°C and 27°C in *Arabidopsis* and therefore has potential to be used as an indicator of ambient temperature perception in plants. The expression of *HSP70* can also be used as an indicator of temperature stress responses in Arabidopsis and rice (Goswami *et al*., 2010; Sung *et al*., 2001).

This chapter investigates whether there are differences in the ability of different wheat cultivars to defend against different pathogens under varying temperature opposed to a constant temperature. Results reveal that basal resistance in cultivar Claire appears to be more able to restrict pathogen colonisation under varying temperature conditions compared to constant temperature regimes in several plant-pathogen interactions. Further investigation reveals insight into timing of pathogen prevention and the suitability of this trait for mapping. The nature of Claire's general response to temperature is also explored.

## 4.3 Methods

### 4.3.1 Plant and pathogen material

See chapter 2 for details on cultivars and pathogen isolates used in this study.

### 4.3.2 Plant growth conditions

Plants were sown in 1 litre pots each with 5 seeds to a pot. Pots were transferred to one of three separate CE cabinets (Sanyo) which were all programmed with 12/12 hr day/night cycles and a constant relative humidity of 80%. The first temperature regime was set to a constant 15°C throughout the 24hr period, hereby referred to as 'constant'. The second regime was programmed to ramp from a minimum of 10°C in the middle of the dark period to a maximum of 20°C in the middle of the light period, hereby referred to as 'varying'. The third cabinet was programmed to ramp from a minimum of 5°C in the middle of the dark period to a maximum of 25°C in the middle of the light period, hereby referred to as 'extreme varying'. Temperature ramping was in 2 hr increments and was an average of 15°C in all CE cabinets. Figure 4.1 shows a simplified diagram of the three temperature regimes.

### 4.3.3 Inoculation of plants

Plants were initially screen using *P. syringae* to enable high throughput of experiments. After initial screening, plants were exposed to a range of UK pathogens to enable investigation of whether defence responses to necrotrophs and biotrophs were affected in the same way. When plants reached three leaf stage, the newest fully developed leaf was inoculated with the various pathogens detailed below. Plants remained in the temperature regimes described above, both pre- and post-inoculation.

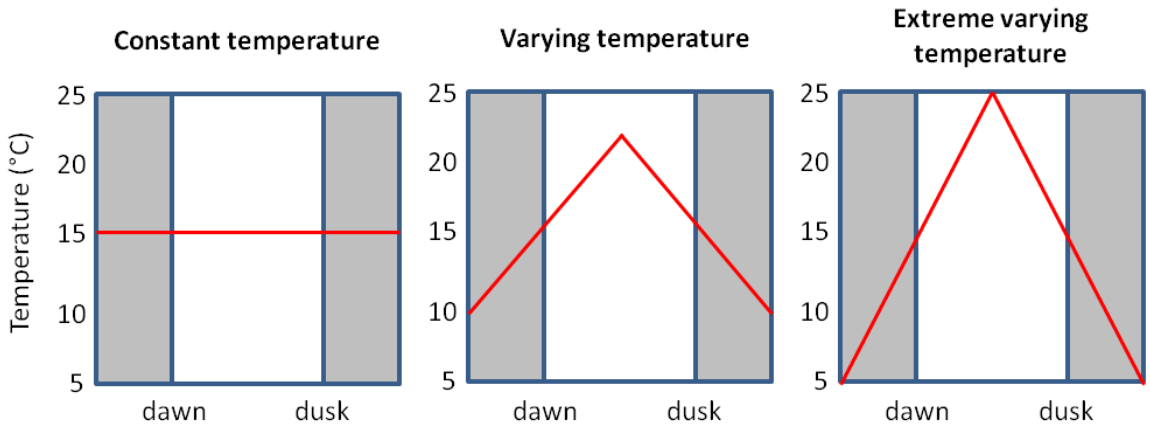



**Figure 4.1 Simplified diagram of the three temperature treatments used in this chapter** CE cabinets (Sanyo) were programmed to run at a 12/12 hr light(white shading)/dark (grey shading) cycle at 80% relative humidity. Red lines indicate CE cabinet temperature through a 24hr period showing at which part of the day minimum and maximum temperatures were reached but in reality temperature increases and decreases were in 2 hr increments.

#### 4.3.3.1 *P. syringae* inoculation

Up to 1 hr pre-inoculation, a loop was taken from a fresh *Por*36_1 culture and re-suspended in sterile dd$H_2O$ with 5% KB media. The $OD_{600}$ of the solution was determined using a spectrophotometer and then diluted to a final concentration of $OD_{600}$ 0.02 for inoculation. Plants were temporarily removed from CE cabinets and leaves were pricked with a pin at five points down the length of the leaf with equal distance between pinpricks.2 µL of the bacterial solution was dropped onto each of the pinpricks and allowed to dry in the CE cabinet, before being placed in a transparent bag with a tray containing water to create a humid environment. At 4 dpi, visual disease symptoms could be observed which enabled selection of two representative lesions to be sampled from each leaf. Leaf tissue was added to an ABgene® 2.2 mL Deep Well Plate (Thermo Fischer Scientific, Loughborough, UK) with 500 µL of KB media and two 5 mm smooth grinding balls. Samples were ground using the GenoGrinder™ (SPEX SamplePrep, LLC) at 1100 strokes $min^{-1}$ for 20 secs. The leaf tissue suspension was diluted serially in KB broth and 10 µL was plated onto KB media containing 25 mg $mL^{-1}$ nystatin and 50 mg/l rifampicin. Plates were incubated overnight at 28°C. Colony number per 10 µL drop was counted from the appropriate dilution and used to calculate colony forming units per lesion.

To assess *in vitro* growth of *P.syringae* in each of the temperature treatments, the same bacterial suspension used for inoculation was added in equal amounts to tubes and placed alongside plants in the different CE cabinets. The $OD_{600}$ of these tubes were measured at various time points over the course of the experiment.

#### 4.3.3.2 *F. culmorum* inoculation

Inoculum was prepared by adding deoxynivalenol at a final concentration of 25 ppm to *F. culmorum* isolate Fu 42 conidia suspended in dd$H_2O$, at a concentration of 0.5 x $10^{-7}$ spores $mL^{-1}$. Inoculation method was adapted from Chen *et al* (2009) to enable of inoculation of attached leaves. Plants were temporarily removed from CE cabinets and leaves were pricked with a pin at five points down the length of the leaf with equal distance between pinpricks. 4 µL of the *F. culmorum* inoculum was dropped onto each of the pinpricks and allowed to dry in the CE cabinet before being placed in a transparent bag with a tray

containing water to create a humid environment. At 5 dpi disease progression was assessed by measuring lesions with a ruler.

To assess *in vitro* growth of *F. culmorum* in each of the temperature treatments, 10 µL of the conidia suspension was dropped onto agar plates containing v8 medium. Growth rate of hyphae was recorded over the course of the experiments by measuring the diameter of the colony.

#### 4.3.3.3 *Bgt* inoculation and microscopy

3.5 cm leaf strip replicates were cut from plants and placed in plastic boxes containing mildew culture media and then placed in metal inoculation towers at room temperature. *Bgt* spores of isolate JIW48 (bulked on leaf strips) were collected and blown on to leaf strips from a height of 50 cm to enable even settling of spores on all leaf surfaces. Boxes were returned to the relevant temperature regime and assessed 6-10 dpi. Leaf strips for microscopic assessment were sampled at 24 hpi and 72 hpi and prepared using the method outlined in section 2.2.4 The progress of the pathogen was determined at both time-points, recording percentage of spores with external hyphae at 24 hp, and the percentage of spores with established hyphae at 72 hpi.

#### 4.3.3.4 *Pst* inoculation

Inoculation of plants was as described in chapter 3, section 3.3.3 for inoculation of seedlings.

### 4.3.4 Measurement of *HSP70* expression

The method originally used for screening wheat for genes involved in temperature perception was adapted from Kumar and Wigge (2010) by Laura Dixon and Adrian Turner (John Innes Centre, Norwich). Plants were grown for two weeks in CE cabinets (Sanyo) set to a 16/8 hr light/dark cycle with a constant temperature of 16°C. One hour following the 15$^{th}$ dawn post sowing, the temperature was dropped to 12°C and leaf tissue for RNA

extraction was sampled after 1 hr. One hour after sampling, the temperature was raised to 27°C. Leaf tissue from different plant individuals was sampled precisely 24hrs after the original sampling time. For the expression profile under constant and varying temperatures, plants were grown for two weeks in CER's set to the same conditions as the CE cabinets used for the pathology studies. Tissue samples were collected at the middle of the dark period, followed by subsequent sampling at 6hrs (beginning of light period), 12 hrs (middle of light period) and 18hrs (beginning of dark period). All tissue was harvested and stored according to section 2.2.5 ready for grinding and RNA extraction from tissue, and subsequent cDNA synthesis in accordance with section 2.2.7. RT-qPCR was conducted on samples to assess *HSP70* expression levels in accordance with section 2.2.7 using primers 4a and 4b in table 2.2 for amplify *HSP70* transcript. A reference gene was selected using the primers in 2.3 to normalise the data as described in section 2.2.8.

### 4.3.5 Data analysis

Where possible, four or more individual plant replicates and more than one experimental repeat were used to calculate means and standard error unless otherwise specified. Locations of plants in the cabinets were randomised differently for each experiment. Pathology assays were analysed with the un-paired *t*-test or general linear regression using the statistical package Genstat for Windows, release 12 (VSN international, Hemel Hempstead, UK). RT-qPCR data were analysed with the un-paired (two sample) *t*-test using the same package.

## 4.4 Results

### 4.4.1 Screening cultivars reveals Claire is more resistant to *P. syringae* under varying temperatures

Wheat cultivars were screened for resistance against *P.syringae* under constant and varying temperatures. Bacterial growth in liquid medium was first measured to determine whether there was a difference in growth between temperature treatments. No significant difference in $OD_{600}$ of bacteria suspended in 5% KB solution was observed between constant and varying temperatures (Figure 4.2a). Out of ten cultivars, only Claire and Pavon

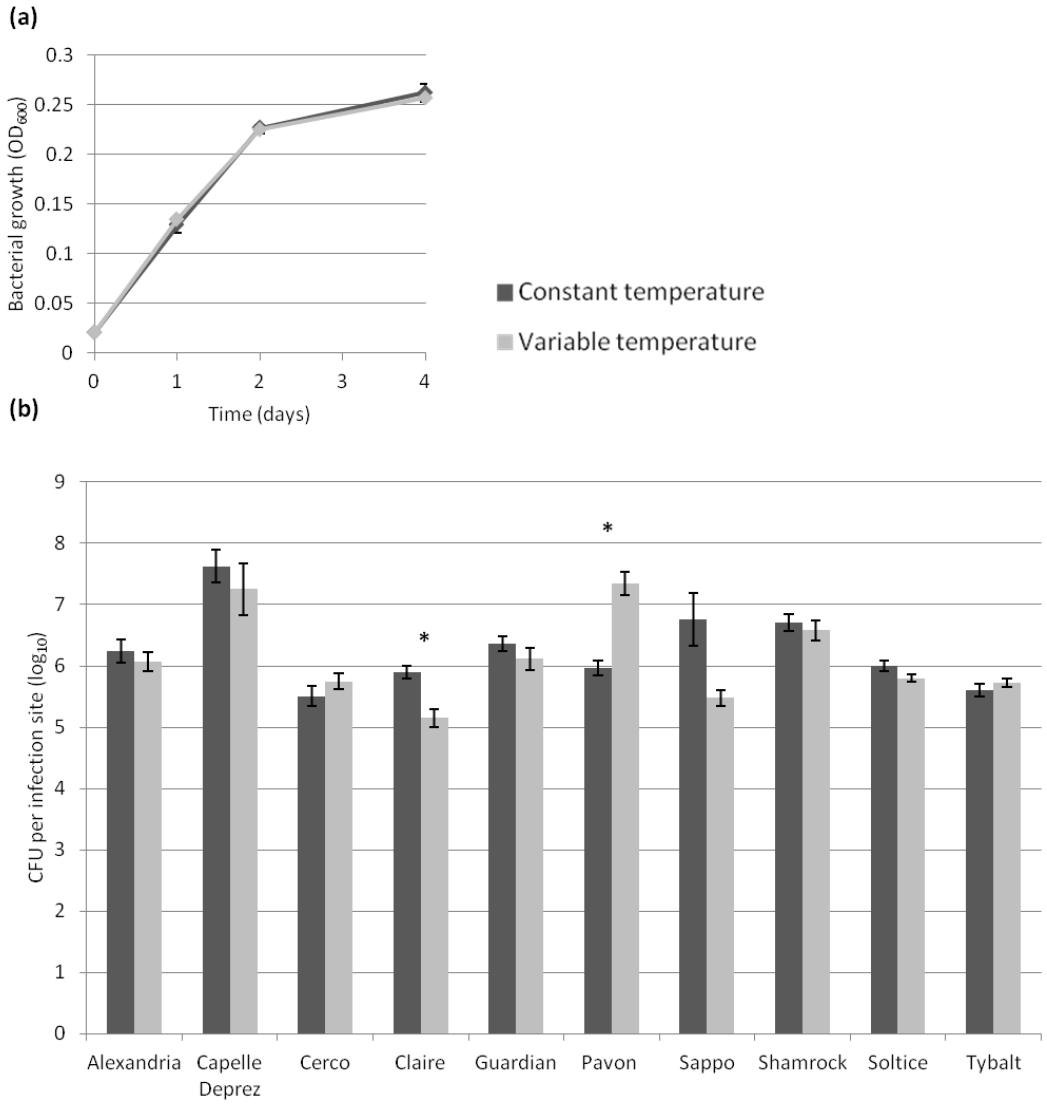



**Figure 4.2 Most wheat cultivars are equally susceptible to *P.syringae* when challenged under constant and varying temperature treatments with the exception of Claire and Pavon. (a)** Tubes containing *P.syringae* ($OD_{600}$ 0.02) suspended in 5% KB medium were placed in each CE cabinet. Bars represent mean $OD_{600}$ readings (± SE) from two biological replicates which were taken over a 4 day period. **(b)** Wheat seedlings were grown at constant and varying temperatures for three weeks, at which point the 3rd leaf was challenged with *P.syringae* ($OD_{600}$ 0.02). At 4 dpi the infection site was sampled and CFU assayed. Bars represent mean CFU from 4 individual plant replicates (± SE). Significant differences between treatments are indicated with an asterisk (*t-test p-value < 0.05*).

had a significant difference in CFU per lesion between plants grown under constant and varying temperatures (Figure 4.2b). In cultivar Claire, significantly less CFUs were observed in plants grown under varying conditions when compared to CFUs formed in plants grown under constant temperatures ($p = 0.03$). Conversely, there were fewer CFUs from Pavon leaf tissue in plants grown under constant conditions compared to those grown under varying conditions ($p = 0.04$, Figure 4.2b).

### 4.4.2 Further investigation reveals Claire has more consistent resistance to *P.syringae* under varying temperatures than other cultivars

To validate the result of the previous experiment and check it wasn't a result of Type I error, the experiment was repeated four times using Claire and Shamrock as a control. CFU counts in Shamrock plants were consistently not significantly different between the two temperature treatments (Figure 4.3). Increased CFU under varying temperatures was not seen again in Pavon plants, although Claire plants had reduced CFU counts by approximately two fold under varying temperatures in two out of four experiments (Figure 4.3). Claire and Shamrock were therefore used in a series of subsequent experiments as examples of cultivars with resistance responses that were affected or unaffected by exposure to varying temperatures.

### 4.4.3 Claire is more resistant to *F. culmorum* under varying temperatures

Shamrock plants showed no significant differences in *F. culmorum* induced lesion size between temperature treatments (Figure 4.4a,b). In Claire plants, lesion size was approximately two fold smaller in plants that experienced varying temperatures compared to those grown under constant temperatures ($p < 0.001$, Figure 4.4a,b). *In vitro* growth of *F. culmorum* was not significantly different between the two temperature treatments (Figure 4.4c). The experiment was not repeated so it has not been determined whether the result is inconsistent as seen with the *P. syringae* interaction.

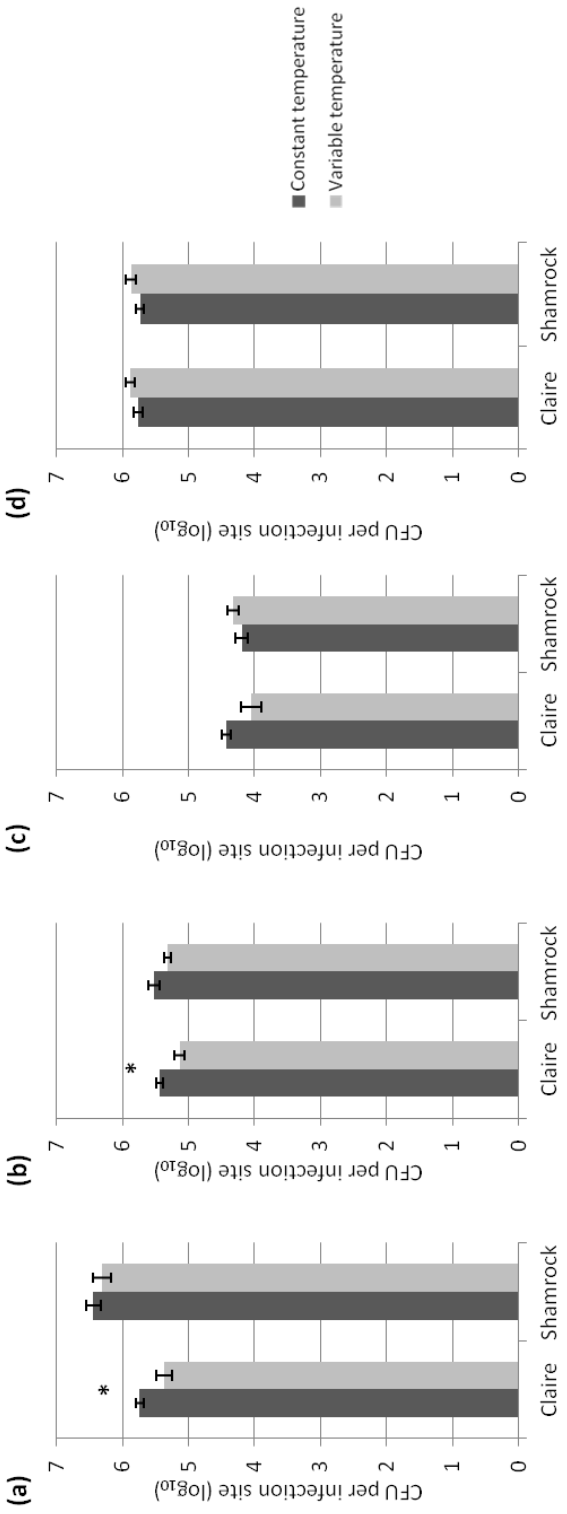



**Figure 4.3 Increased resistance to *P.syringae* under varying temperatures is not always seen in wheat cultivar Claire.** Wheat seedlings were grown at constant and varying temperatures for three weeks, at which point the 3rd leaf was challenged with *P.syringae* ($OD_{600}$ 0.02). At 4 dpi the infection site was sampled and CFU assayed. The experiment was repeated several times **(a)** 1st repeat, **(b)** 2nd repreat, **(c)** 3rd repeat and **(d)** 4th repeat. Bars represent mean CFU from five individual plant replicates (± SE). Significant differences between treatments are indicated with an asterisk (*t-test p-value < 0.05*).

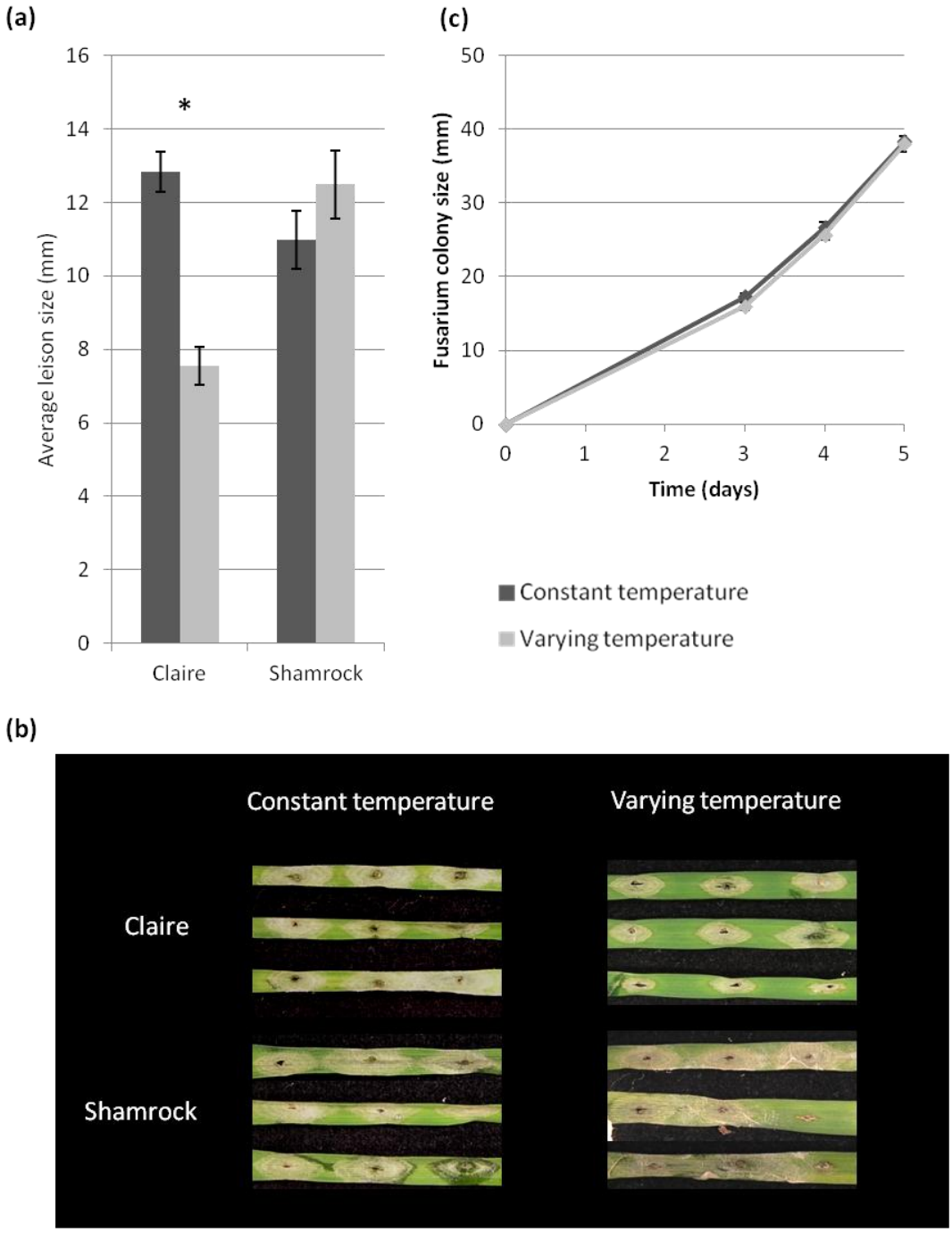



**Figure 4.4 Wheat cultivar Claire is more resistant to *Fusarium* when grown under a varying temperature. (a)** Wheat seedlings were grown at either constant or varying temperatures for three weeks, at which point the 3rd leaf was inoculated with *Fusarium* and the resulting lesion size was measured 5 dpi. Bars represent mean size of (± SE) of *Fusarium* lesions from 10 or more lesions per five plant replicates. Significant differences between treatments are indicated with an asterisk (*t-test p-value < 0.001*). **(b)** *Fusarium* visual symptom development at 5 dpi in cultivars Claire and Shamrock when grown and inoculated under constant or varying temperatures. **(c)** *Fusarium* spores suspended in water were plated onto agar plates containing V8 media and the size of the colony was measured over the course of the experiment. Data points are the mean of 2 plate reps (± SE ).

### 4.4.4 Claire is more resistant to *Bgt* under varying temperatures

When plants were inoculated with *Bgt* in the two different temperature regimes, the number of colonies that developed on Shamrock leaves was not significantly different between the two temperature regimes (Figure 4.5a,b). Claire plants showed a reduced number of mildew colonies under variable temperatures every 2 in 3 experiments, the maximum differences being two fold. Predicted means generated using general linear regression showed there was a significant difference overall ($P < 0.01$, Figure 4.5a,b).

### 4.4.5 Increased resistance to *Bgt* under varying temperatures in Claire can be seen as early as 24 hpi

When enhanced resistance under varying temperatures was seen in Claire plants, colonisation and progression of mildew was observed microscopically. At 24 hpi Shamrock plants in the two temperature regimes did not have a significantly different percentage of mildew spores that were able to form external hyphae (Figure 4.6a,c). A similar observation was made in Shamrock at 72hrs with no significant difference in percentage of established hyphae between plants grown under constant and varying temperatures (Figure 4.6b,c). Claire on the other hand had significantly fewer spores forming external hyphae in plants grown under varying conditions ($P = 0.03$, Figure 4.6a,c). Claire plants tended towards a reduced percentage of established hyphae in plants under varying conditions but variation was high and mildew establishment in the two temperature treatments was not significant at the 95% confidence interval ($P = 0.11$ Figure 4.6b,c).

### 4.4.6 Claire shows an avirulent response when challenged with *Pst* isolate 08/21

To determine whether there is a link between the observations in chapter 3, Claire and Shamrock were challenged with *Pst* isolate 08/21. Pustules did not develop on Claire when grown under a constant temperature and a necrotic response was apparent on plants grown at constant temperatures confirming that Claire has one or more *R* genes that can recognise this isolate (Figure 4.7). Interestingly a significantly higher level of pustules developed on Claire grown under varying temperatures, ($P < 0.01$). In Shamrock, pustule coverage was significantly higher on plants under varying temperatures compared to

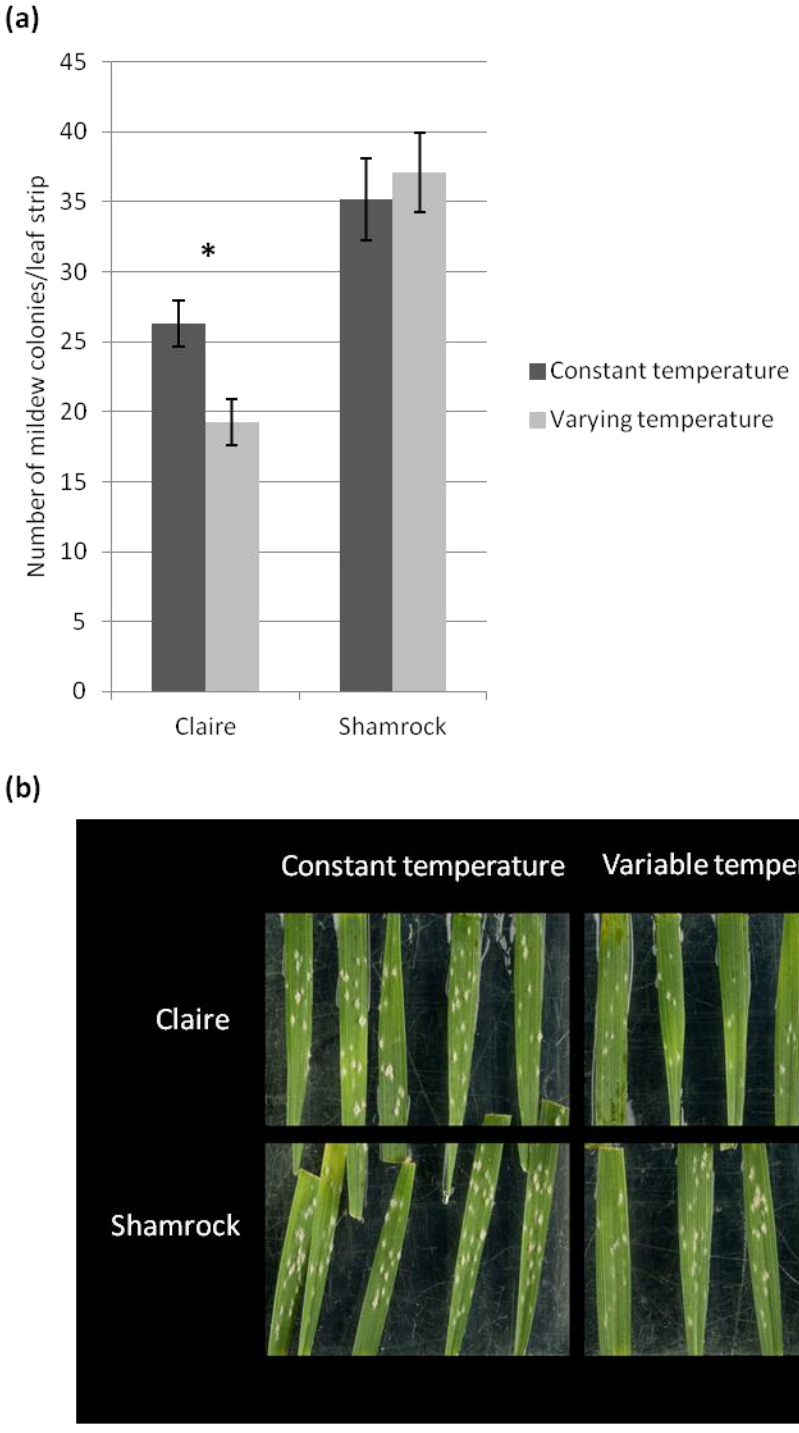



**Figure 4.5 Wheat cultivar Claire can be more resistance to mildew when grown under varying temperatures. (a)** Wheat seedlings were grown at either constant or varying temperatures for three weeks, at which point leaf strips were sampled from the 3rd leaf and inoculated with mildew. Bars represent number of mildew colonies per leaf strip at 8 dpi and are predicted means (± 1 SE) from three independent experiments using the general liner model. Significant differences between treatments are indicated with an asterisk ($P < 0.01$). **(b)** Mildew visual symptom development at 8 dpi in cultivars Claire and Shamrock when grown and inoculated under constant or varying temperatures from a representative experiment.

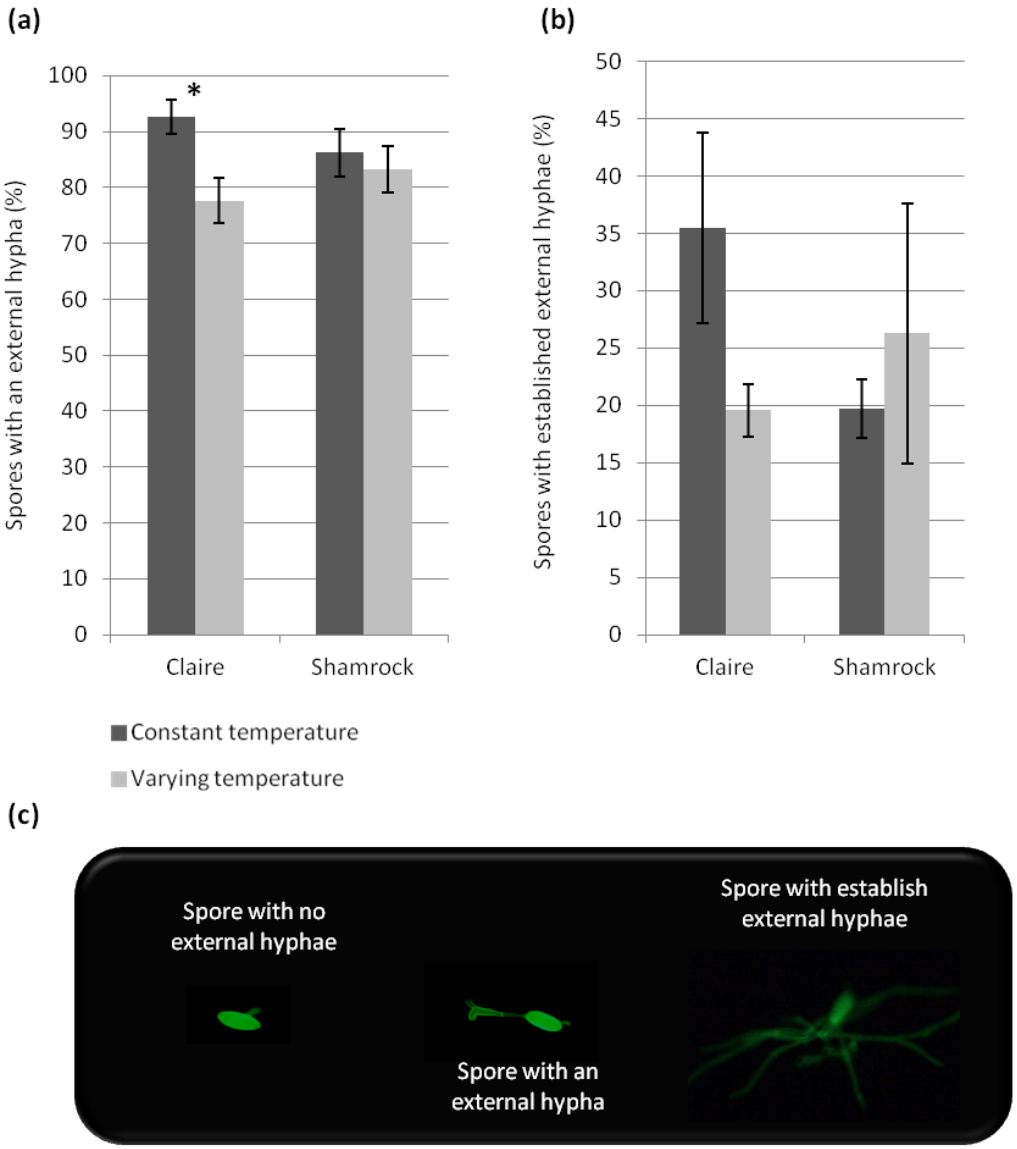



**Figure 4.6 Enhanced resistance in Claire due to varying temperature can be seen 24hrs after inoculation.** Wheat seedlings were grown at either constant or varying temperatures for three weeks, at which point leaf strips were sampled from the 3rd leaf and inoculated with mildew. **(a)** 24hrs after inoculation, samples were prepared for microscopy and pathogen progression was observed. Bars represent mean number of spores with an external hypha (± SE) from four independent plant individuals. Significant differences between treatments are indicated with an asterisk ($P < 0.05$). **(b)** Pathogen progression was observed at 72 hrs post inoculation. Bars represent mean number of spores with established external hyphae (± SE) from four independent plant individuals. Significant differences between treatments are indicated with an asterisk ($P < 0.05$). **(c)** Visual development of mildew pathogen as seen using a florescence microscope (Nikon 800 Eclipse) which was used to score pathogen progression at 24 hrs and 72 hrs post inoculation.

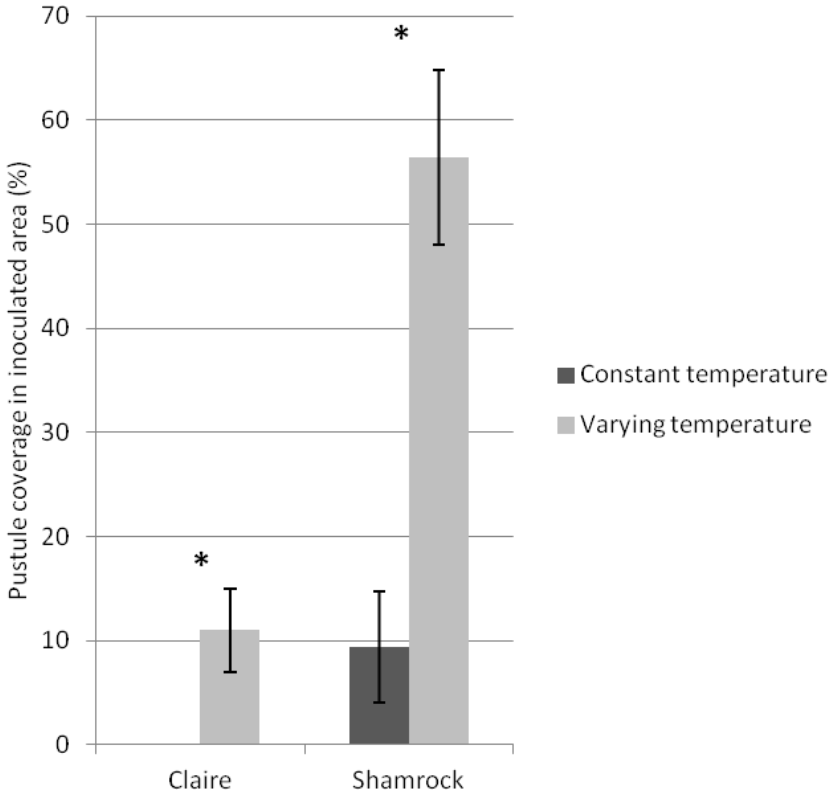



**Figure 4.7 Yellow rust development in Claire and Shamrock after being challenged with *Pst* isolate 08/21 under constant and varying temperatures.** Wheat seedlings were grown at either constant or varying temperatures for three weeks, at which point the 3rd leaf was inoculated with yellow rust isolate 08/21. Bars represent mean leaf pustule cover at 18 dpi in inoculated area (± 1 SE) from five plant replicates. Significant differences between treatments are indicated with an asterisk (*t-test p-value < 0.01*).

pustule coverage on plants maintained at constant temperatures, which was in contrast to results observed for all other pathogen challenges (Figure 4.7).

### 4.4.7 No effect of temperature treatment is seen on resistance in Claire or Shamrock to *Pst* isolate 11/08

Due to Claire having an *R* gene for gene resistance response to *Pst* isolate 08/21, cultivars were also tested with *Pst* isolate 11/08 to see whether disease development was different between the two temperature regimes. No difference in pustule coverage was observed between plants exposed to a constant temperature compared to those exposed to varying temperatures in neither Claire nor Shamrock (Figure 4.8).

### 4.4.8 Exposing plants to a more extreme variable temperature environment affects resistance differently depending on the pathogen

Using both *P.syringae* and *Bgt*, the effect of exposing the plants to a more extreme varying temperature on plant defence was determined. Mildew colony numbers were significantly reduced on Claire under varying temperatures compared to plants at constant temperatures as previously seen. However when plants experienced more extreme varying temperatures, the number of mildew colonies was significantly higher than those seen in both of the other temperature treatments ($P < 0.01$, Figure 4.9a). In Shamrock there was no significant difference between the number of mildew colonies that developed on the leaf strips in all three temperature treatments. When the same experiment was performed using *P.syringae*, *in vitro* bacterial growth rate was measured as before. $OD_{600}$ of *P.syringae* grown at constant temperatures was slightly less compared to the other temperatures treatments after one day of incubation but there were no significant differences in $OD_{600}$ between treatments at four days (Figure 4.9b). CFU counts in Claire appeared lower in plants grown under varying temperatures compared to those grown at constant temperatures but the difference was not quite significant at a 95% confidence level ($P = 0.058$, Figure 4.9c). CFU counts in Claire plants under more extreme varying temperatures also appeared lower than in plants at constant temperatures but again the difference was

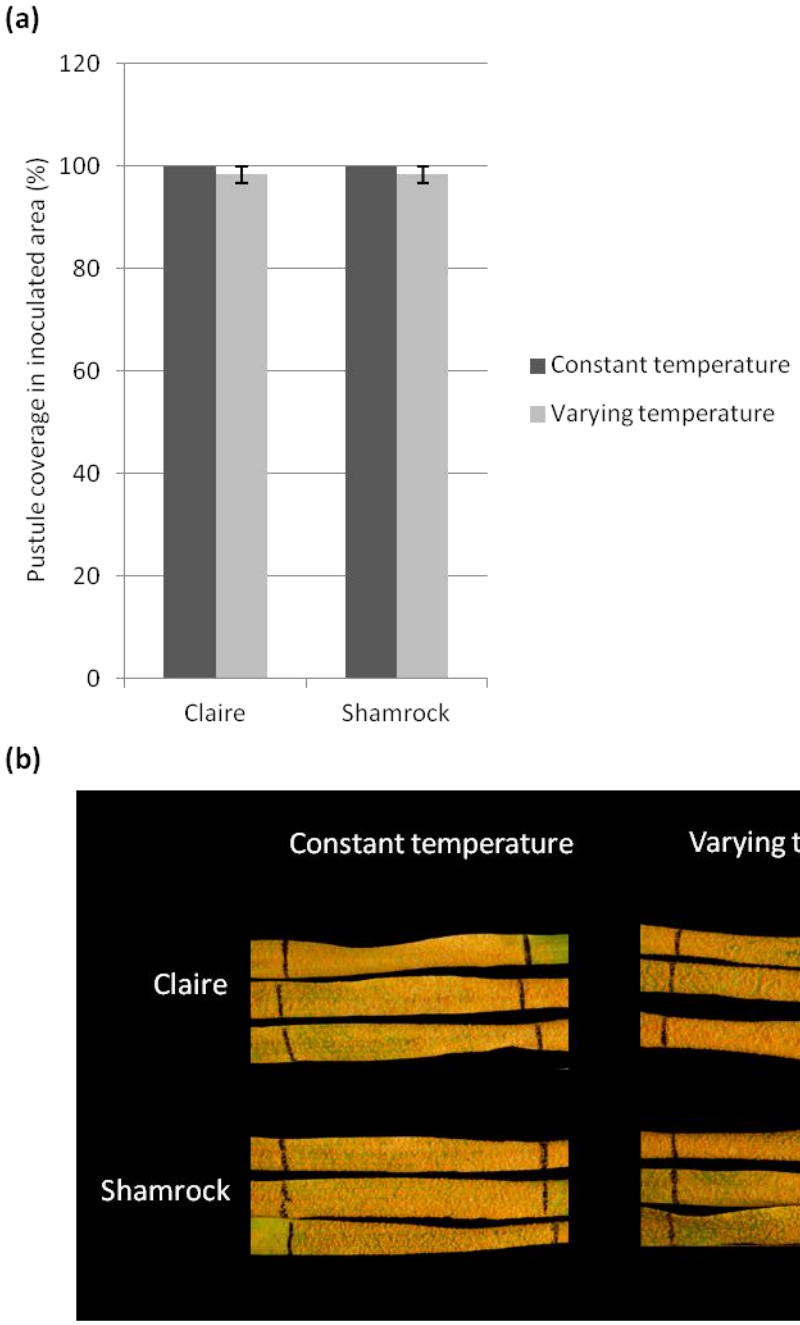



**Figure 4.8 No affect of temperature treatment is seen on resistance in Claire or Shamrock to *Pst* isolate 11/08.** Wheat seedlings were grown at either constant or varying temperatures for three weeks, at which point the 3rd leaf was inoculated with yellow rust isolate 11/08. **(a)** Bars show mean pustule coverage (± 1 SE) in the inoculated area at 18 dpi from three plant replicates. Significant differences between treatments are indicated with an asterisk (*t-test p-value < 0.05*). **(b)** *Pst* symptom development at 18 dpi in cultivars Claire and Shamrock when grown and inoculated under constant or varying temperatures.

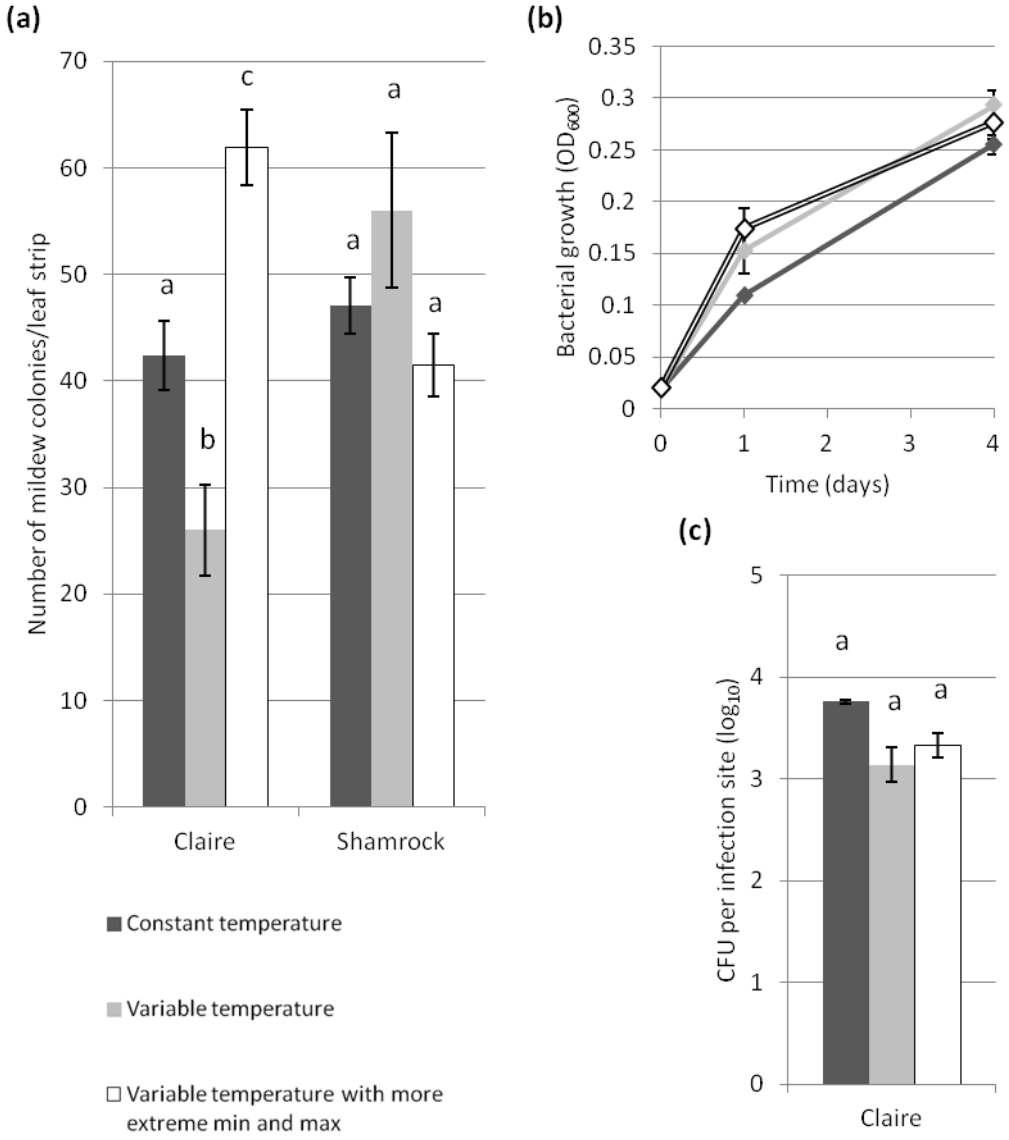



**Figure 4.9 Increasing the difference between the min and max temperature indicates that resistance to different pathogens is affected in different ways. (a)** Wheat seedlings were grown at either constant, varying and more extreme varying temperatures for three weeks, at which point leaf strips were sampled from the 3rd leaf and inoculated with mildew. Bars represent mean number of mildew colonies per leaf strip (± 1 SE) from five individual plant replicates at 8 dpi. Data was analysed using general linear regression and different letters indicate significant differences between treatments within a genotype (*t-test p-value < 0.05*). **(b)** Tubes containing *P.syringae* ($OD_{600}$ 0.02) suspended in 5% KB medium were placed in each CE cabinet. Bars represent mean $OD_{600}$ readings (± 1 SE) from two biological replicates which were taken over a 4 day period. **(c)** Wheat seedlings were grown at constant, varying and more extreme varying temperatures for three weeks, at which point the 3rd leaf was challenged with *P.syringae* ($OD_{600}$ 0.02). At 4 dpi the infection site was sampled and CFU assayed. Bars represent mean CFU from 4-5 individual plant replicates (± 1 SE). Data was analysed using general linear regression and different letters indicate a significant difference between treatments (*t-test p-value < 0.05*).

not significant (*P=0.11*, Figure 4.9c). Shamrock plants were not infected so could not be used as a control in this experiment.

### 4.4.9 *HSP70* transcript levels in Claire are more sensitive to temperature changes than in Shamrock

To ascertain whether there was any difference between ambient temperature perception between wheat cultivars Claire and Shamrock, *HSP70* transcript level was measured at two different temperatures. When Claire plants were transferred from 12°C to 27°C within a 24 hr period, the fold change between *HSP70* transcript levels between the two temperatures was significantly higher than the fold change seen in Shamrock (*P < 0.01*, Figure 4.10a). Comparison of the relative expression of *HSP70* transcript indicates that the reason for the larger fold change in Claire is due to low levels of *HSP70* transcript at 12°C compared Shamrock, which was approximately ten fold lower (Figure 4.10b), whereas at 27°C, HSP70 transcript levels did not differ significantly between the two cultivars (Figure 4.10c).

### 4.4.10 Exploratory expression profile of *HSP70* transcript in Claire and Shamrock under constant and varying temperatures

To link results obtained using the method adapted from Kumar and Wigge (2010) to the present study, *HSP70* transcripts were compared between the two cultivars in the two different temperatures regimes. Results indicate that under constant temperatures, *HSP70* transcripts in both Claire and Shamrock increased over the 24hr period from the middle of the dark period to the end of the light period and transcript levels were higher in Shamrock than in Claire (Figure 4.11a). Under varying temperatures the steady incline of *HSP70* transcript over the 24hr period was not seen in either variety (Figure 4.11b). In the middle of the dark period (time 0hrs on Figure 4.11b), transcript abundance was 35 fold higher in Shamrock compared to Claire and over ten-fold higher than transcript levels observed at any other time point in either variety (Figure 4.11b).

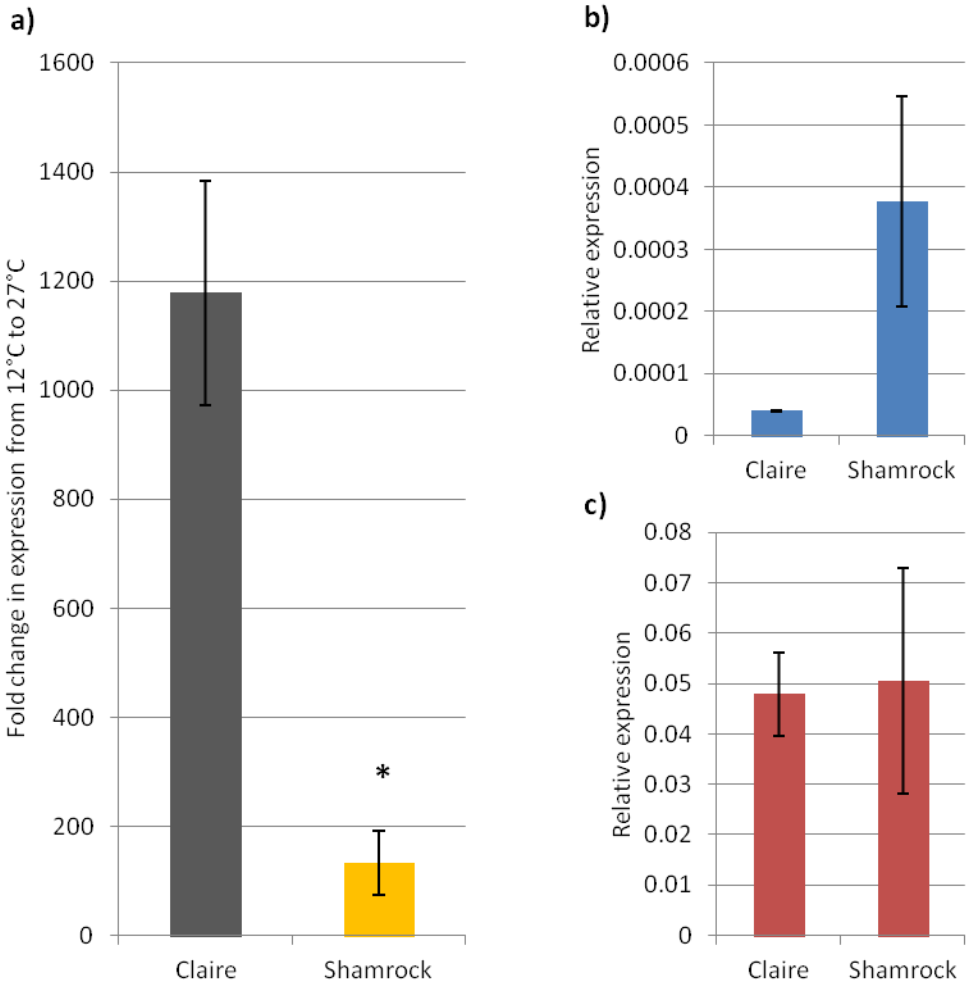



**Figure 4.10 HSP70 expression change in Claire is more extreme than in Shamrock when plants are moved from 12°C to 27°C.** Plants were grown at 16°C for 2 weeks before the temperature was dropped to 12°C for 1 hr before leaf samples were taken for RNA expression analysis. Plants were then transferred to 27°C and sampled 24 hrs later. **(a)** HSP70 transcript expression fold change between plants transferred from 12°C to 27°C in cultivars Claire and Shamrock. Bars represent mean fold change (± 1 SE) from two experiments during which transcript levels were normalised to elongation factor-1 α. Significant differences between cultivars are indicated with an asterisk (*t-test p-value < 0.01*). **(b)** HSP70 transcript expression at 12°C. Bars represent mean relative expression (± 1 SE) calculated from two experiments each containing one biological replicate which consisted of pooled leaves from 3-4 different plants. **(c)** HSP70 transcript expression at 27°C. Bars represent mean relative expression (± 1 SE) calculated from two experiments with four biological replicates, each containing pooled leaves from 3-4 different plants.

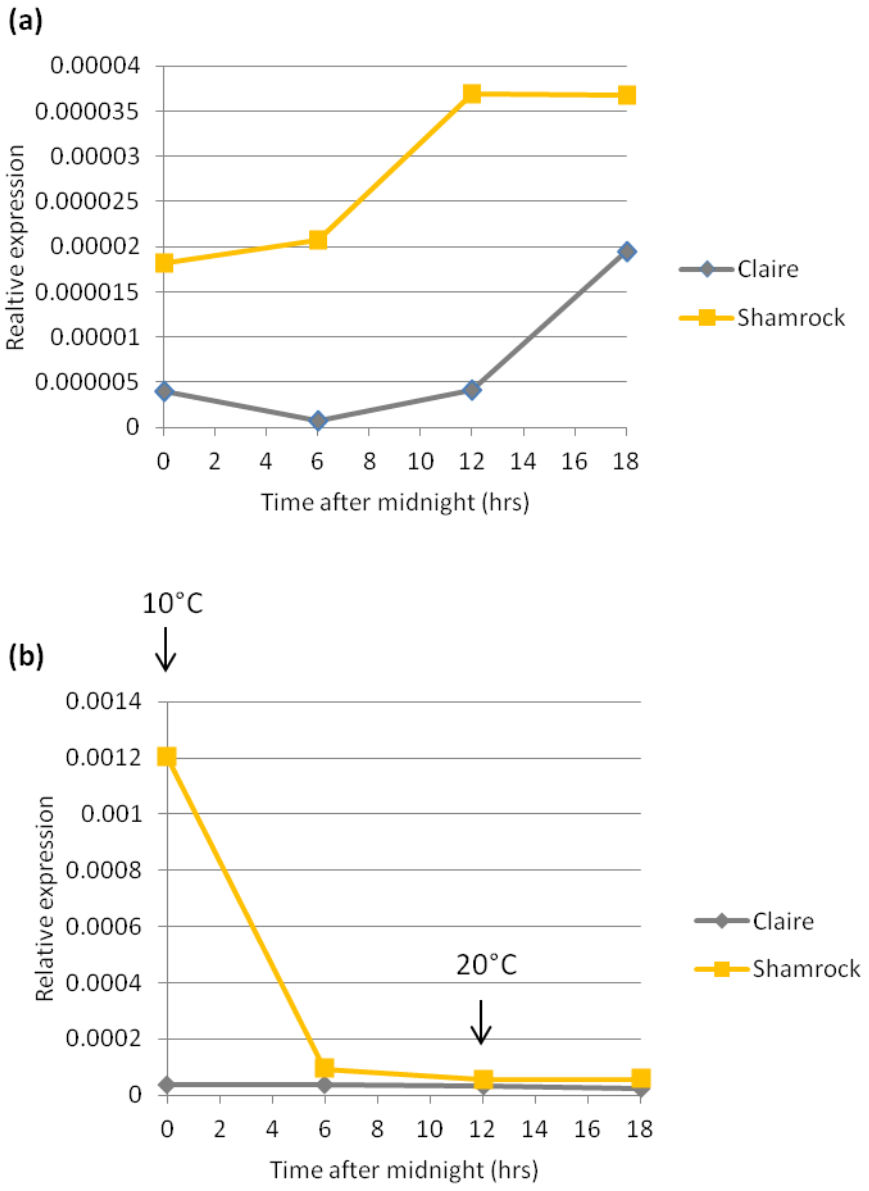



**Figure 4.11 Exploratory 24 hr HSP70 expression profile in plants grown under constant and varying temperatures** Plants were grown under constant or varying temperature for 2 weeks before leaf samples were taken for RNA expression analysis from the middle of the dark period to the end of the light period. HSP70 transcript expression profiles were constructed for cultivars Claire (grey diamonds) and Shamrock (yellow squares) grown under **(a)** constant temperatures or **(b)** varying temperatures. Data points represent transcript levels from one RNA extraction, pooled from three individual plant replicates and transcript levels are normalised to elongation factor-1 α. High and low temperature peaks are indicated.

## 4.5 Discussion

When possible to measure, *in vitro* pathogen growth under constant and varying temperatures was the same, suggesting that pathogen growth is not affected by differences in thermoperiods provided the average temperature remains the same. Therefore, results suggest that cultivar Claire can show increased resistance or reduced susceptibility under varying temperatures in the four plant-pathogen interactions in this study. This is in contrast to defence in cultivar Shamrock which was generally at a similar level under constant and varying temperatures. Thus, the effect is due to a host response rather than influencing pathogen development, which was a concern in the previous chapter. The initial screen using *P. syringae* showed that increased resistance under varying temperatures in Claire might be an exception in modern wheat varieties. If this is the case then this trait could be a useful tool for breeding considering it may help protect plants against pathogen invasion under the frequent temperature changes present in field conditions. However, results reveal that defence in Claire plants exposed to increased varying temperatures between 5°C and 25°C is no more effective than plants exposed to varying temperatures between 10°C and 20°C. In fact, in the wheat-mildew interaction, the highest levels of mildew were seen under the most extreme varying temperatures suggesting that defence is reduced under these conditions. This may be due to the extreme changes triggering host stress that diminishes resistance to powdery mildew. Alternatively this may be due to temperature conditions being more favourable to mildew, although observations from Shamrock suggest otherwise as mildew levels were not significantly different between all three temperature conditions. Therefore, if Claire does have increased resistance under varying temperatures, observations suggest there is a threshold on this resistance. In addition, it cannot be ruled out that increased resistance in Claire may also be caused by an aspect of resistance that can only function above or below a threshold of 15°C rather than the variation in temperature causing the increased resistance. There are many examples of the literature of small differences in temperature being capable of switching on or off resistance mechanisms (Whitham *et al*., 1996; Xiao *et al*., 2003; Yang and Hua, 2004). However these thresholds are usually associated with *R* gene for gene interactions and HR which is not the type of resistance that has been observed here. A contrasting study by Koga *et al* (2004a) saw that a low temperature treatment of 10°C inhibited a novel type of resistance in rice plants to *Magnaporthe grisea* that was independent of a blast resistance gene (Koga *et al*., 2004b). They showed that *de novo* ABA

biosynthesis in the leaf sheaths was responsible for the reduction of resistance. This is clearly not the case in Claire plants, as exposure to 10°C does not reduce resistance. However, it would be interesting to measure levels of plant hormones that are involved in plant defence in Shamrock and Claire under constant and varying temperatures to see if there are possible candidates that are involved in the enhanced resistance observed in Claire.

Microscopic observations with mildew suggest that early defence in Claire is being affected by the temperature variation. This could be because varying temperature is affecting i) innate immunity in this cultivar or ii) development of the plants so pathogens can invade tissue less easily. Results indicate that the increased resistance in Claire under varying temperature is not restricted to one type of pathogen and occurs across kingdoms, implying that a type of plant defence which conveys resistance to a broad spectrum of pathogens such as PTI. It is possible that PTI response in Claire may be strengthened by a 10°C difference in thermoperiod or that the component is only functional above or below a threshold of 15°C.

It is well documented that increased difference between day and night temperature increases stem elongation and subsequently plant height (Berghage and Heins, 1991; Erwin *et al*., 1989; Grimstad and Frimanslund, 1993). If an increased thermoperiod is increasing plant growth, it is possible that this is indirectly affecting resistance. Although not measured, there were no obvious indications that there were differences in growth between wheat plants grown under varying compared to constant temperatures. As discussed in chapter 3, there is evidence that plants balance resources between growth and defence to achieve maximum productivity (Mosher *et al*., 2010). However if Claire plants are balancing resources in favour of growth under varying conditions more so than in Shamrock, then it is having a positive effect on defence responses rather than antagonising them. GAs have been implicated in the difference observed between stem elongation in several plants species under different thermoperiods due to reduction in differences after exogenous application of GA (Grindal *et al*., 1998; Ihlebekk *et al*., 1995; Zieslin and Tsujita, 1988). In addition, endogenous levels of some GAs are higher in plants experiencing varying temperatures opposed to constant temperatures (Stavang *et al*., 2005). When GA accumulates, DELLA proteins are degraded which could explain increased resistance in Claire plants to biotrophic pathogen *Bgt*, but not increased resistance to

necrotrophic pathogen *F. culmorum*. A more rational explanation is that varying temperature is affecting development of pre-formed physical or chemical barriers in Claire plants.

Since its release in 1999, Claire has been used as a parent in many breeding programs due to its high yield and durable resistance to numerous diseases (Powell *et al*., 2013). Disease resistance in this cultivar has been durable since its release with the exception of yellow rust, which was originally complete and broke down in 2012, and brown rust which was almost complete and broke down in 2005 (Figure 4.12a). For *Fusarium*, mildew and *Septoria* diseases, resistance has always been quantitative. It is tempting to speculate that the durable QDR in Claire to all pathogens is due to this cultivar being more able to convey resistance under varying temperatures compared to other cultivars. It will be interesting to see whether the newly broken yellow rust resistance in Claire will be maintained at a QDR level considering it was the only pathogen observed here that did not show increased resistance in Claire plants under varying temperatures in CE experiments. However the experiment was only done once, which makes it difficult to say whether increased resistance to *Pst* is never seen due to the variable phenotype observed with the other pathogen types. Claire's yield, relative to the control cultivar, has declined over the years since its release but not enough for the cultivar to be removed from the HGCA Recommended List (Figure 4.12b). It would be interesting to know whether Claire is generally better at dealing with temperature variation which indirectly makes it more able to deal with pathogen attack and/or other stresses, simultaneously to temperature changes.

Results showed that *HSP70* expression levels in both Claire and Shamrock was higher at 27°C, with the fold difference higher in Claire probably due to the low expression level at 12°C. Transcript expression of *HSP70* genes have been shown to increase under environmental stress conditions such as heat, cold and drought stress in several plant species (Goswami *et al*., 2010; Sung *et al*., 2001). A role for *HSP70* in cold acclimatisation in addition to heat stress has also been implicated (Zhang *et al*., 2008). If *HSP70* transcript abundance is an indicator of stress, results suggest that Shamrock plants are more stressed at 12°C than Claire plants at the same temperature due to higher expression levels in Shamrock plants. A first-look expression profile of *HSP70* transcript levels in the constant and varying temperature regimes revealed that Shamrock showed extremely high levels of

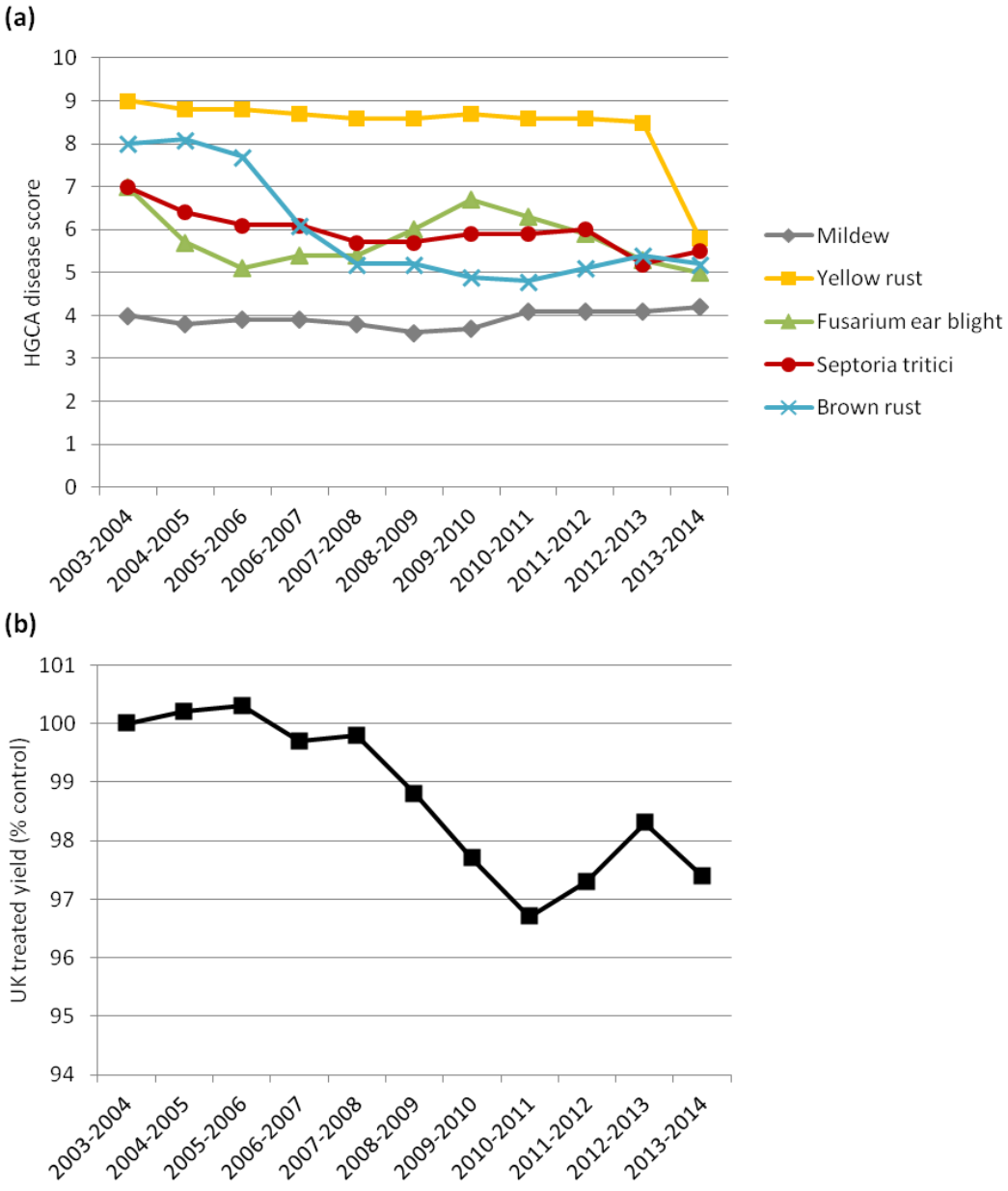



**Figure 4.12 Disease scores and yield of Claire over the last decade.** **(a)** Disease scores of Claire taken from HGCA Recommended Lists from 2003 to 2013. Data points represent resistance score for a given disease: 1 = totally susceptible 9 = Totally resistant. **(b)** Claire UK treated yield taken from HGCA Recommended Lists from 2003 to 2013. Data points represent % yield relative to control cultivar Solstice.

*HSP70* transcripts at 10°C, again suggesting that this cultivar might be experiencing more stress than Claire at lower temperatures. This stress clearly does not affect resistance in Shamrock, considering a difference in pathogen abundance between the two temperature regimes was rarely observed in this variety. However it is tempting to speculate that the lack of stress observed in Claire under varying temperatures could somehow be related to the enhanced resistance observed. If a stress response wasn't triggered in Shamrock at 10°C under varying temperature conditions, would enhanced resistance be seen in this cultivar too? Using the method adapted from Kumar and Wigge (2010), *HSP70* transcript at 12°C was always expressed at a lower level in Claire compared to all other varieties suggesting that other varieties may be experiencing an elevated level of stress at this temperature (Figure 4.13).

In chapter 3, Shamrock resistance was classed as having a defence response to *Pst* that was sensitive to a temperature change. When challenged with *Pst* isolate 08/21 using the temperature treatments in this chapter, *Pst* levels were significantly lower on plants that were grown under constant temperatures opposed to varying temperatures. It is difficult to compare the two experiments as plants didn't experience a change in temperature for the first time, at the time of inoculation in this chapter. In addition, when Shamrock plants were challenged with a different UK yellow rust isolate, *Pst* levels were the same under both regimes suggesting that the temperature sensitivity observed in both chapters 3 and 4 may be limited to the Shamrock-08/21 interaction. The fact that Solstice and Shamrock showed the same level of resistance in both temperature regimes during the initial screen also suggests that the temperature sensitive nature of cultivars between the two experimental methods cannot be linked.

The next step towards identifying potential genes controlling the response to changing temperatures would be to screen mapping populations between Claire and a non temperature sensitive variety. However, due to inconsistency of results, this trait would not be easy to map. Results from both chapters 3 and 4 suggest that temperature sensitive resistance is extremely sensitive to other factors such as light levels, humidity and water availability. Further work would have to be done to define the environmental conditions so that results were more reproducible and mapping populations could be screened successfully.

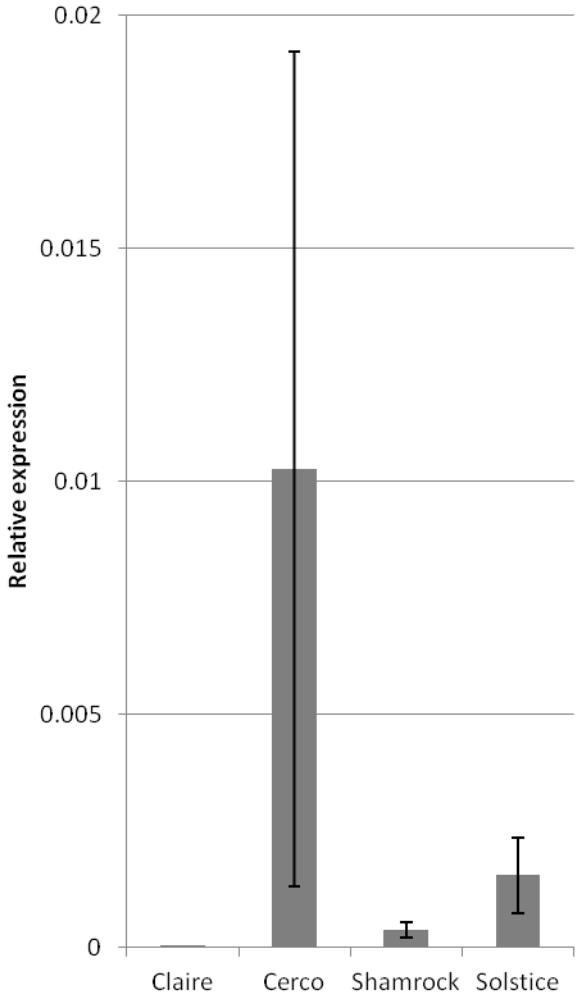



**Figure 4.13 Comparison of HSP70 transcript expression between cultivars.** Plants were grown at 16°C for two weeks before the temperature was dropped to 12°C for 1 hr before leaf samples were taken for RNA expression analysis. Bars represent mean relative expression of HSP70 transcript expression at 12°C (± 1 SE), calculated from two biological replicates each from an independent experiment containing pooled leaves from 3-4 different plants. Transcript levels were normalised to elongation factor -1 α.

# Chapter 5 Temperature effects on PRR expression and using microarray data to identify genes that are differentially regulated by both a temperature change and upon disease challenge

## 5.1 Aim

Experiments conducted in this chapter were carried out to investigate the basis for how temperature changes affect disease resistance in wheat. Preliminary investigations were carried out to determine whether the effects of temperature changes on disease resistance are based on PAMP-triggered immunity (PTI), the first line of active defence in plants.

## 5.2 Introduction

Results presented in chapter 3 and 4 indicated that a temperature change can affect wheat resistance to pathogens, although it is unclear which components of the defence mechanisms are affected. It was observed that a reduction in temperature is able to enhance susceptibility to *Pst* and unexpectedly, an increase in temperature is able to enhance resistance. This is contrary to general observation that temperature increases are usually associated as being detrimental to plant defence responses (Szittya *et al*., 2003).

Zhu *et al* (2010) argue that NB-LRR types of *R* genes are responsible for temperature sensitivity rather than other signalling components. However results presented throughout chapters 3 and 4 suggest otherwise, considering that temperature sensitivity in Claire affects resistance against widely diverged microbial taxa. As discussed in the introduction, PTI is the earliest active defence in plants, induced by conserved molecules present across taxa. It is a reasonable hypothesis that altered PTI responses induced by temperature changes may be the basis for the observations in chapter 4. This chapter explores whether aspects of wheat defence are sensitive to temperature reductions, focusing primarily on PTI.

Chapter 1 outlines how PTI occurs when essential PAMPs are detected by specific plant PRRs (Zipfel, 2009). Several PAMP-PRR pairs have been described in several plant species including crops, for example FLS2 which detects the derived peptide flg22 subunit of flagellin in various bacterial pathogens (Robatzek *et al*., 2007; Takai *et al*., 2008) and

CERK1/CEBiP can pair with chitin which is specific to fungal pathogens (Shimizu *et al*., 2010; Shinya *et al*., 2012). When the respective PAMP-PRR pairs converge, a MAPK signalling cascade is initiated, which leads to a defence response (Schwessinger and Ronald, 2012; Zipfel, 2009). The preliminary experiments designed here, were performed to determine whether there is any evidence that PTI is affected by temperature changes using an extreme temperature reduction as a starting point. Results show that PRR transcripts are affected by a temperature reduction but not overall resistance.

Due to wheat PRR transcripts being affected by temperature reductions, it was logical to determine whether other wheat defence transcripts were also affected. Data from various microarray experiments were analysed to attempt to identify whether there are clusters of genes in wheat that are involved in both plant acclimatisation to a reduction in temperature and broad spectrum defence. No gene clusters were identified as responding to both temperature and general plant defence.

## 5.3 Methods

### 5.3.1 Plant and pathogen material

Wheat line UC1041 + *Yr36* was used in all qRT-PCR and pathology assays. *P. syringae* strain *Por*36_1 and *Bgt* isolate JIW48 were used in all pathology assays. See chapter 2 for details on wheat line and isolate details and maintenance.

### 5.3.2 Growth conditions and cold treatment

Plants were grown in a CE cabinet (Sneijder) at a constant 20°C with a 16 hr/8hr day/night cycle. Two weeks after sowing, plants were either kept at the same conditions or exposed to a cold night by being moved to an identically programmed CE cabinet set to 5°C in darkness. Tissue samples were harvested from the 2$^{nd}$ leaf 1 hr and 4 hrs after the start of the dark period (according to section 2.2.5) ready for RNA extraction from tissue, followed by subsequent cDNA synthesis in accordance with section 2.2.7. qRT-PCR was conducted on samples to assess *CEBiP* and *FLS2* transcript expression levels in accordance with section

2.2.7 using primers 5a, 5b, 6a and 6b and reference genes was selected using the primers in 2.3 to normalise the data as described in section 2.2.8.

### 5.3.3 Inoculation with *P.syringae*

4 hrs after the start of the dark period, two-week old plants were inoculated from both control and cold treated plants. Using scissors, plants were scored longitudinally in a 4 cm adaxial region of the leaf and then dipped in bacterial solution for 30 secs. Plants were kept in polythene bags to encourage bacterial growth and returned to the original temperature regime of 20°C (regardless of preceding control or cold treatment). At 3 dpi plants were removed from growth cabinets and two leaf discs were taken from the inoculation site on each plant using a 4 mm diameter core borer. Leaf discs were added to an ABgene® 2.2 mL Deep Well Plate (Thermo Fischer Scientific) with 500 µL of KB media and two 5 mm smooth grinding ball. Samples were ground using the GenoGrinder™ (SPEX SamplePrep, LLC) at 1100 stokes $min^{-1}$ for 20 secs. The leaf tissue suspension was diluted serially in KB broth and 10 µL was plated onto KB media containing 25 mg $mL^{-1}$ nystatin and 50 mg/l rifampicin. Plates were incubated overnight at 28°C. Colony number per 10 µL drop was counted from the appropriate dilution and used to calculate CFU/$cm^2$ of tissue.

### 5.3.4 Inoculation with *Bgt*

As above, two-week old plants were inoculated 4 hrs after the start of the dark period. 4 cm leaf strip replicates were cut from plants and placed in plastic boxes containing mildew culture media and placed in metal inoculation towers at room temperature. *Bgt* spores from bulk plants were collected and blown onto leaf strips from a height to enable even settling of spores on all leaf surfaces, then left to settle for 5 minutes before being returned to the original temperature regime of 20°C (regardless of preceding control or cold treatment). The number of mildew colonies per leaf strips was recorded at 10 dpi.

### 5.3.5 Data analysis

For experiments measuring PRR transcript abundance, three experimental repeats (each comprised of three pooled plant individuals) were used to calculate means and standard error unless otherwise specified. RT-qPCR data were analysed with the un-paired (two sample) *t*-test using the statistical package Genstat for Windows, release 12. Transcript abundance was compared independently at each time point.

### 5.3.6 Microarray processing and analysis

Affymetrix datasets for an experiment carried out by Laudencia-Chingcuanco *et al* (2011), investigating cold induced genes, were downloaded from PLEXdb (http://www.plexdb.org/modules/PD_probeset/annotation.php) along with datasets from various experiments involving wheat-pathogen interactions. Genes identified from Laudencia-Chingcuanco *et al* (2011) with differential expression (>4 fold) in all wheat cultivars, between cold treatment of 6°C for 48 hrs and their respective control of 0 hrs was calculated using linear modelling and an Empirical Bayes moderated *t* statistic (Smyth, 2004). 232 genes were commonly differentially expressed by cold treatment at the 24hr time point among all varieties used in the study (see appendix for list of genes). The expression profile of those probe sets was exported into a tab delimited file along with the expression profile of the same probe sets from the pathogen induced experiments also available from the PLEXdb database (Bolton *et al*., 2008; Bozkurt *et al*., 2010; Coram *et al*., 2008a; Coram *et al*., 2008b; Desmond *et al*., 2008; Jia *et al*., 2009; Tufan *et al*., 2009; Xin *et al*., 2011). Hierarchical clustering was performed using Cluster 3 (Eisen *et al*., 1998) with a Euclidean distance matrix and complete-linkage clustering technique.

## 5.4 Results

### 5.4.1 A cold night changes expression patterns of PRR transcripts

To determine whether temperature had a direct affect on PRR transcript abundance, plants were first exposed to a temperature drop in absence of a pathogen challenge. qRT-PCR showed that UC1041 plants, kept at a constant 20°C showed increased expression of *CEBiP* transcript during the dark period after 1 hr, which become more apparent after 4 hrs of darkness (Figure 5.1a). *FLS2* transcript showed a similar trend to *CEBiP*, upon the onset of

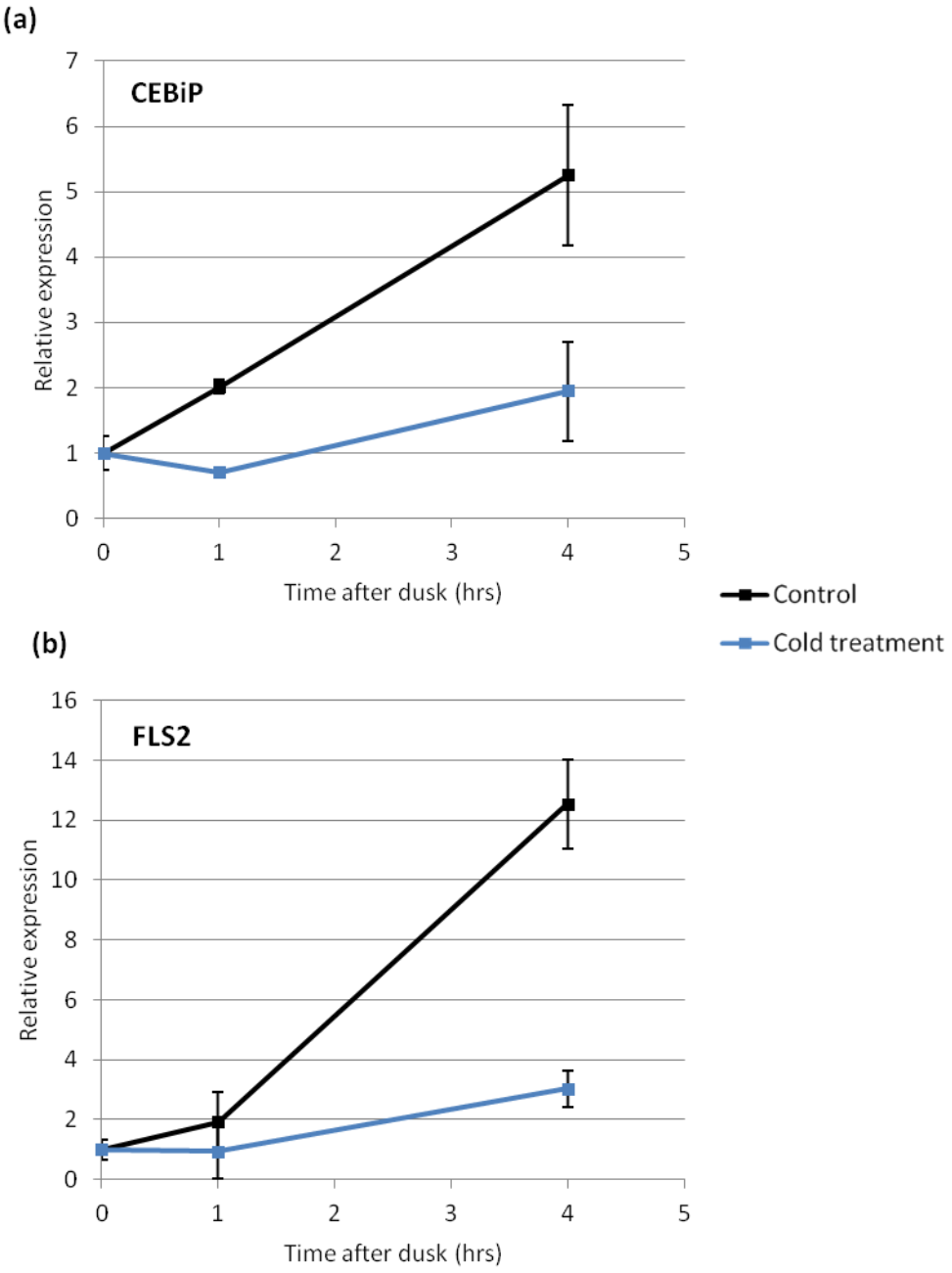



**Figure 5.1 Relative expression of PRR genes in newest fully developed leaf when plants are exposed to a cold night.** Plants were grown for two weeks before either remaining at a constant 20°C or being exposed to a cold treatment of 5°C from the beginning of the dark period (dusk). RNA was extracted from 2nd leaf and analysed for expression of PRRs using RT-qPCR **(a)** *CEBiP* expression profile calculated relative to the 0 hr time point (set to 1). Data points show mean relative *CEBiP* expression levels from three independent experiments, each of pooled plant material from three biological replicates (± 1 SE). Transcript levels are normalised to elongation factor-1 α and ubiquitin. **(b)** Equivalent values for *FLS2* transcripts.

the dark period (Figure 5.1b). When plants are exposed to a 5°C cold treatment during the night, *CEBiP* transcript levels do not increase during the dark period, resulting in a difference of more than twofold at both 1 hr and 4 hrs, between control plants and cold treated plants (Figure 5.1a). *FLS2* transcripts in cold treated plants are not significantly different from the control after 1 hr, however at 4 hrs post cold treatment control plants have 4 fold more *FLS2* transcript abundance than cold treated plants (Figure 5.1b).

### 5.4.2 Differential expression of PRRs induced by cold night does not affect over-all plant resistance when plants are challenged at midnight

To see whether the difference in PRR transcript abundance at 4 hrs between the two treatments had an effect on plant defence, plants were inoculated with both *Bgt* and *P. syringae*. Pathogens were chosen because being a fungal pathogen, powdery mildew naturally contains chitin (Zhang *et al*., 2000) which is detected by PRR CEBiP (Shimizu *et al*., 2010). Contrastingly, *P. syringae* contains flg22 detected by FLS2 (Zipfel *et al*., 2004). When plants were inoculated with *Bgt* at 4hrs post cold treatment they did not have a significantly different level of mildew colonies from control plants at 10 dpi (Figure 5.2a). A similar trend was seen when plants from both treatments were infected with *P. syringae* in that 3 dpi, bacterial levels were not significantly different between control or cold treated plants (Figure 5.2b).

### 5.4.3 Identification of genes involved in both temperature perception and disease resistance

To identify clusters of genes that were differentially expressed by both a temperature change and broad spectrum pathogen challenge, hierarchical clustering was performed with various data sets downloaded from PLEXdb. No gene clusters were identified (Figure 5.3).

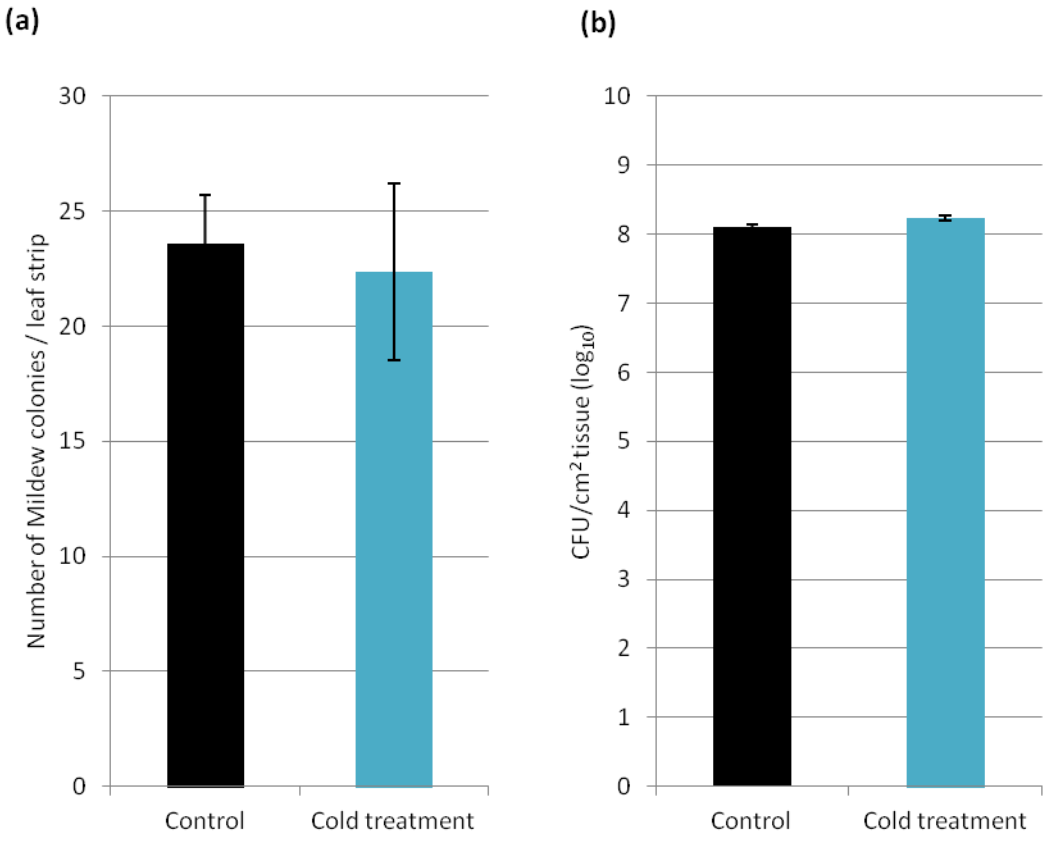



**Figure 5.2 Effect of exposing plants to a cold night on resistance to *Bgt* and *P. syringae*.** Plants were grown at a constant 20°C for two weeks, at which point the plants were either kept at the same temperature at the beginning of the dark period (black bars) or transferred to 5°C (blue bars). 4 hrs post treatment the 2nd leaf was challenged with pathogens **(a)** 4 cm leaf strips were challenged with *B. graminis* isolate JIW48. Bars show mean mildew colonies per leaf strip (± 1 SE), calculated from 3 biological replicates from one experiment that is representative of two independent experiments. All plants were returned to a constant 20°C after inoculation and disease score was conducted 10 dpi. **(b)** 4 cm leaf strips were challenged with *P. syringae*. Bars show mean CFU/cm² of tissue per leaf strip (± 1 SE), calculated from 3 biological replicates from one experiment that is representative of two independent experiments. All plants were returned to a constant 20°C after inoculation and disease score was conducted 3 dpi.

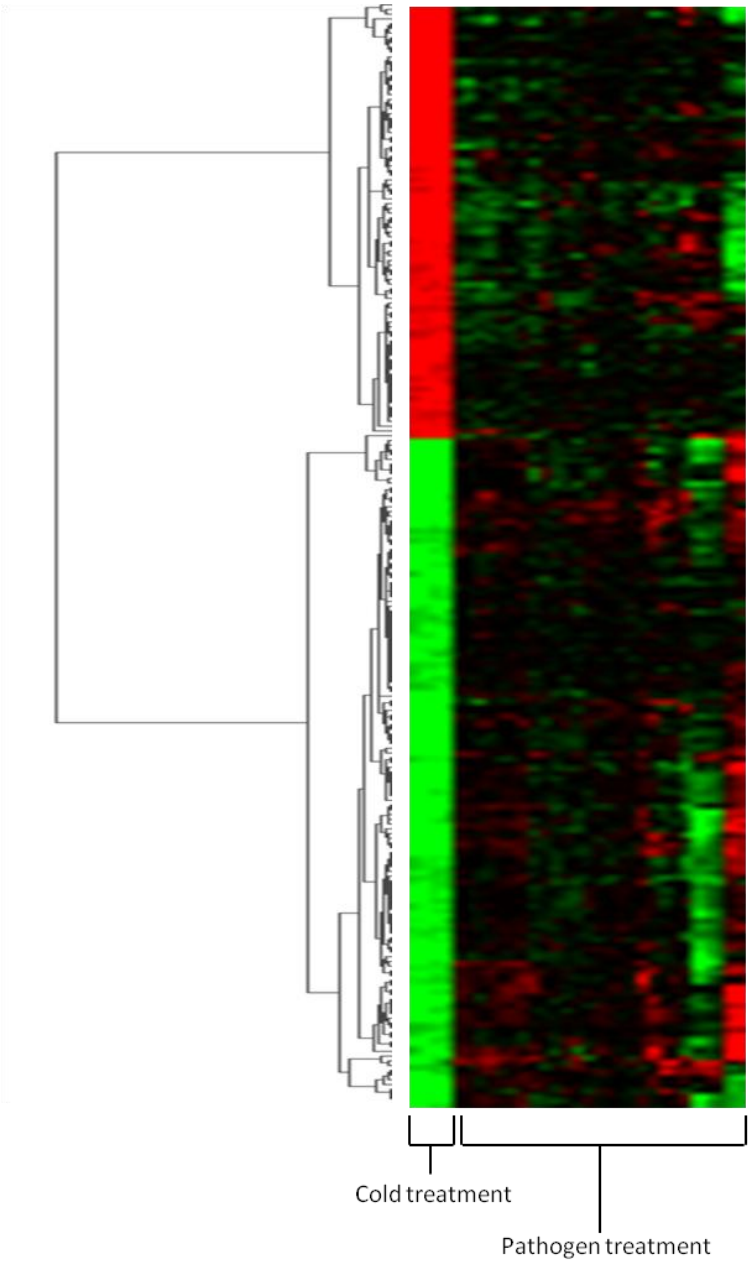



**Figure 5.3 Hierarchical clustering of probe sets differentially expressed upon cold treatments with expression of the same probe sets when challenged with various pathogens.** Meta-analysis of probe sets on the Affymetrix Wheat GeneChip differentially transcribed in response to cold and the same probe sets' response to pathogen challenge. Experiments included are low temperature treatment of 6°C for 48 hrs (Laudencia-Chingcuanco *et al.,* 2011), *Magnaporthe oryzae* inoculations with isolates BR32 or BR37 and *M. grisea* isolate BR29 (Tufan *et al.*,2009), incompatible and compatible interactions with *Pst* (*Yr5, Yr39* and *Yr1*; Coram *et al.*, 2008a,b; Bozkurt *et al.*, 2010), incompatible and compatible interactions with *P. triticina* (*Lr34* and *Lr1*; Bolton *et al.*, 2008), and compatible *Bgt* (Xin *et al.*, 2011) and *Fusarium pseudograminearum* and *F. graminearum* (Desmond *et al.*, 2008; Jia *et al.,* 2009) inoculations. The colour scale represents $\log_2$ expression values of up regulated (red blocks) and down regulated (green blocks) genes.

## 5.5 Discussion

### 5.5.1 Effect of night temperature on expression pattern of PRRs

The preliminary experiments described here were designed to investigate whether temperature changes affected PTI as the basis for the observations made in the previous chapters. As a baseline for comparison, PRR gene expression was first determined, revealing that for plants grown at a constant temperature, FLS2 and CEBiP have a diurnal expression pattern. This is consistent with Bhardwaj *et al* (2011) which shows that Arabidopsis PRRs exhibit a diurnal expression pattern and are regulated by the circadian clock. Results shown here indicate that when plants are exposed to a cold night, amplitude of the diurnal rhythm is reduced.

The evidence presented here indicates that a temperature change can affect PRR expression, but that this does not affect disease resistance. When plants were challenged with pathogens *Bgt* and *P. syringae* 4 hrs post dusk, no difference in eventual disease levels was observed between plants kept at a constant temperature and those that had been exposed to a cold night, despite the difference between PRR transcripts. There are a number of possibilities to explain why a difference was not observed. Firstly, mRNA levels do not always represent the level of protein present in the cell and many transcripts show diurnal rhythm patterns but the proteins levels do not change throughout the day (Tian *et al*., 2004). However as variations in expression patterns of PRRs have already been shown to influence plant defences at different times of day, it is plausible that protein levels are also changing (Bhardwaj *et al*., 2011). It is well documented that when PAMPs come into contact with PRRs that a defence response is initiated (Pitzschke *et al*., 2009), although the timing of these processes during plant-pathogen interactions is poorly understood. Another reason could be that a threshold may exist where increased PRR abundance provides enhanced resistance. If this is not achieved at the point at which pathogen challenge first occurs, no difference in resistance would occur. If the plants were challenged with the bacteria at an earlier time point, the increased abundance of PRRs at midnight in control plants may have aided with defence. However in Arabidopsis, *FLS2* induction by *P. syringae* DC3000 can be detected as early as 2hrs post-inoculation (de Torres *et al*., 2003). The disease assays performed did not discriminate between PTI and other stages of resistance such as ETI and subsequent basal resistance, therefore the other stages of the defence response could have masked any differences between PTI responses in control and cold

treated plants. There is very limited evidence to date that PTI responses are affected by temperature. Arabidopsis proteins PAD4 and EDS1 show temperature sensitivity and contribute to both basal defence mechanisms in addition to R gene mediated defence (Wang *et al*., 2009). However it is not known whether they play a role in PTI directly.

### 5.5.2 Identification of genes involved in both temperature perception and disease resistance

Results identified that no clusters of genes were induced both during disease resistance and after cold treatment. This could imply that the two are not co-regulated. However, the analysis was based on previously published data from several experiments with differences in sampling times, methods of inoculation and wheat varieties used, which would reduce the possibility of detecting common gene clusters induced by both treatments. In Arabidopsis there have been examples of proteins that are directly affected by temperature and resistance but studies to date have mainly focused on high temperature inhibition of defence responses (Wang *et al*., 2009; Zhu *et al.,* 2010). However there is also evidence to suggest that low temperature can induce resistance responses in wheat due to biotic and abiotic stress responses having common pathways (Ergon *et al*., 1998; Kuwabara *et al*., 2002). Increased resistance is thought to be through induction of pathogenesis-related (PR) proteins brought on by the cold treatment (plants were exposed to temperatures of 4°C or less) which primes plants for pathogen attack (Ergon *et al*., 1999). Additional work by Szechynska-Hebda *et al* (2013) showed that cold hardening can also prevent pathogen penetration though physical and chemical alterations of the leaf surface properties, interestingly in a cultivar dependent manner. In contrast low temperature has also been linked to susceptibility of rice to rice blast fungus caused by *Magnaporthe grisea,* implying a role for ABA has also been implicated. However the low temperature treatment used in the study by Koga *et al* (2004a) was 10°C, so not quite indicative of cold hardening.

Although the *in silico* analysis of gene transcription did not identify co-regulated genes, the results showed that PRR expression can be affected by a temperature change. To develop the study, an experiment needs to be carried out to specifically investigate candidate genes which are commonly differentially expressed by temperature changes and upon pathogen challenge. As PRR and other resistance gene expression can be regulated diurnally,

different time points need to be considered. The approach taken could be through whole transcriptome analysis. Although a reference wheat genome is not currently available, such an investigation could provide insight into the gene classes that may be co-regulated by pathogen and temperature. Such an investigation could contribute to fundamental understanding of temperature effects on resistance in wheat, and enable the identification of genes affecting temperature sensitivity traits in breeding.

# Chapter 6: Effect of temperature on take-all development

## 6.1 Aim

The research presented in this chapter was undertaken with the aim of identifying favourable weather conditions for take-all development from historical data and assessing what data would need to be available to combine with future climate scenarios from *UKCP09* in order to model how take-all disease will be affected by climate change.

## 6.2 Introduction

Chapters 3, 4 and 5 explored the effects of temperature on resistance as a means to identify possible ways of adapting crops to likely climate change. As outlined in Chapter 1, there is no known resistance against take-all disease, making it difficult to build greater temperature resilience through breeding. As such, this chapter explores a different approach to preparing crops for climate change, by forecasting whether a specific disease will become more problematic in the UK so that management practices can be adjusted accordingly.

As briefly outlined in chapter 1, take-all is an important disease of cereal crops caused by the root-infecting, necrotrophic fungus *Ggt*. Estimates predict that up to half of UK wheat crops are affected with yields suffering between 5-20 % losses, costing farmers up to £60 million per year (HGCA, 2006). The disease is divided into a primary and secondary phase of infection, the primary infection being the transfer of inoculum from the soil to the root and the latter being root to root transfer (Hornby and Bateman, 1998). Winter wheat is usually sown in September/October; primary *Ggt* infection takes place between October and March, while secondary infection doesn't occur until the spring. Therefore the predicted increase in UK mild winters might encourage increased primary infection of *Ggt* in the autumn/winter and therefore lead to a more aggressive secondary infection in spring. Previous research has shown that *Ggt* inoculum is able to develop where soil temperatures are between 5° and 30°C, however, severe infections are restricted to soil temperatures between 5° and 15°C (Hornby and Bateman, 1998). Management of the disease is largely through seed treatments and cropping systems. The pathogen has a wide host range including many wild grasses as well as cultivated species, however it does not survive well

in soils absent of host plants. Therefore one of the most effective methods of controlling take-all is through crop rotation, allowing the soil to be periodically free from *Ggt* hosts (Colbach *et al.*, 1997; Ennaifar *et al*., 2007). 2nd and subsequent wheat crops will generally produce less yield than a 1st crop by as much as 2 tonnes per hectare, with loss due to take-all being the main reason for this (Jones 2009). Despite these figures, according to a recent CropMonitor (https://secure.fera.defra.gov.uk/cropmonitor) survey, 25-30% of wheat grown in the UK is in 2nd wheat or more and another source thinks this figure is closer to 40% (Hammond-Kosack, 2011; Jones, 2009). An alternative method used to control take-all is through continual growth of the same cereal crop taking advantage of a phenomenon known as take-all decline. The severity of the disease initially increases over the first few growing seasons but is often followed by a suppression in subsequent crops; a decline due to development of antagonist microbial community in the soil (Gutteridge *et al.,* 2006; Weller *et al*., 2002). Take-all decline doesn't normally set in until a 4th wheat crop and management using this method will normally produce less yield than a 1st wheat (HGCA 2006). Different soil types have different risks associated with take-all risk for example crops on light sandy soils, chalky downland soils and fen peats are all high risk (Catt *et al*., 1986; HGCA 2006). Therefore there are several factors that affect take-all development and the disease will be managed accordingly, depending on the region and grower specific approach.

UKCP09 is introduced in chapter 1 as a public online database that can output future climate predictions in the UK. It is a project designed to meet the needs of a wide range of people that are interested in assessing the potential impact of future climate change in the 21st century. The user interface can be used to generate various maps, graphs and spreadsheets containing projections about an array of environmental variables within the UK, under various scenarios at a regional level (with a resolution of 25km$^2$). UKCP09 also provides access to a series of daily and hourly future climate projections at a greater resolution of 5km$^2$, which is provided by a weather generator (developed by the University of East Anglia). The predictions are based on various future greenhouse emission scenarios which are selected from the IPCC Special Report on Emissions Scenario (IPCC, 2000). Each scenario represents a different storyline defining social-economic driving forces which are key determinants of the future emissions pathway and all scenarios are based on the assumption that emissions will not be changed in response to concerns over climate change (Murphy *et al*., 2009). Previous *UK climate projections* exist, the most recent prior

to UKCP09 being UKCIP02. Projections from UKCP09 were used due to being the most up to date publically available data.

In the work presented in this chapter, the climatic factors important in development of take-all disease are considered. The results indicate that growing subsequent wheat crops in some regions may become a bigger issue under climate change scenarios. Hypotheses about why climatic factors in particular periods are more important than in others are presented and the potential of UKCP09 for making predictions about take-all is also evaluated.

## 6.3 Methods

### 6.3.1 Historical data

Take-all incidence and co-located weather measurements have both been recorded at Rothamsted experimental station since the 1970s, and the local weather has also been monitored for over 100 years. Taking advantage of this, a literature search was conducted to obtain this information and to subsequently analyse it to enable comparison between take-all incidence and climate variables. Rothamsted meteorological records were obtained from the *electronic* Rothamsted Archive (e-RA) and take-all records are summarised in Table 6.1. Spink *et al* (2004) provided an additional dataset of take-all recordings collected over consecuative years from an Agricultural and Environmental Consultancy (ADAS UK, Ltd) site at Rosemaund, Herefordshire. To compare take-all incidence with climate variables in this region, climatic data was obtained from the weather station situated at Great Malvern which is approximately 16 miles from the Rosemaund site. Trials for this data set were grown in the same location each year. A 3$^{rd}$ set of data was obtained from Monsanto, UK Ltd and NIAB consisting of consecutive years' worth of take-all records from several UK sites (Table 6.2). In order to analyse this data, climatic records were obtained from the nearest respective weather stations located at Wattisham for Suffolk trials, Charterhall for Northumberland, Coningsby for Lincolnshire and Andrewsfield for Essex. Trials for this data set were grown within a 20 mile radius. All data sets were analysed for relationships between mean temperatures and rainfall and take-all incidence in spring using a regression analysis. Spring take-all incidence was used for analysis as these data was more widely

**Table 6.1 Origin of data points used in Rothamsted climate/take-all analysis**

| Data Source | Location | Cultivar | Soil | Seed treated | Wheat rotation | Date sown | Year sown | Year of harvest | Date of spring assessment | Plants infected (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| Bateman 1984 | Rothamsted exp farm | Avalon | silty clay loam | unkown | 2nd | 03-Oct | 1980 | 1981 | 15-Apr | 50 |
| Bateman 1984 | Rothamsted exp farm | Avalon | silty clay loam | unkown | 4th | 15-Oct | 1982 | 1983 | 05-Apr | 73 |
| Bateman 1984 | Rothamsted exp farm | Flanders | silty clay loam | unkown | 3rd | 18-Oct | 1979 | 1980 | 15-Apr | 37 |
| Bateman 1984 | Rothamsted exp farm | Avalon | silty clay loam | unkown | 3rd | 27-Oct | 1981 | 1982 | 19-May | 39 |
| Bateman et al, 1993 | Rothamsted Long Hoos | Avalon | flinty clay loam soils | Ceresol | 4th | 15-Oct | 1985 | 1986 | 21-Apr | 8.6 |
| Bateman et al, 1993 | Rothamsted Long Hoos | Avalon | flinty clay loam soils | Gammasan | 3rd | 17-Oct | 1986 | 1987 | 06-Apr | 44.5 |
| Bateman et al, 2004 | Rothamsted Long Hoos IV field | Hereward | unkown | Fluquinconazole | 4th | 22-Sep | 1999 | 2000 | 07-Apr | 91.6 |
| Bateman et al, 2004 | Rothamsted Long Hoos IV field | Hereward | unkown | Fluquinconazole | 2nd | 01-Oct | 1997 | 1998 | 16-Mar | 10.6 |
| Bateman et al, 2004 | Rothamsted Long Hoos IV field | Hereward | unkown | Fluquinconazole | 3rd | 12-Oct | 1998 | 1999 | 12-Apr | 35.8 |
| Bateman et al, 2008 | Rothamsted exp farm | Claire | silty clay loam | Fluquinconazole | 3rd | 16-Sep | 2004 | 2005 | 12-Apr | 93.9 |
| Bateman et al, 2008 | Rothamsted exp farm | Claire | silty clay loam | Fluquinconazole | 4th | 17-Sep | 2004 | 2005 | 20-Apr | 87.8 |
| Bateman et al, 2008 | Rothamsted exp farm | Claire | silty clay loam | Fluquinconazole | 2nd | 23-Sep | 2002 | 2003 | 01-May | 52.9 |
| Bateman et al, 2008 | Rothamsted exp farm | Claire | silty clay loam | Fluquinconazole | 3rd | 14-Oct | 2003 | 2004 | 15-Apr | 51.8 |
| Gutteridge and Hornby, 2003 | Rothamsted little Knott field | Mercia | silty clay loam over clay-with-flints | untreated | 2nd | 12-Sep | 1991 | 1992 | April | 36 |
| Gutteridge and Hornby 2003 | Rothamsted little Knott field | Mercia | silty clay loam over clay-with-flints | untreated | 3rd | 15-Sep | 1992 | 1993 | March | 65.4 |
| Gutteridge and Hornby 2003 | Rothamsted little Knott field | Mercia | silty clay loam over clay-with-flints | untreated | 4th | 24-Sep | 1993 | 1994 | April | 54.8 |
| Gutteridge and Hornby 2003 | Rothamsted little Knott field | Mercia | silty clay loam over clay-with-flints | untreated | 2nd | 14-Oct | 1991 | 1992 | April | 14 |
| Gutteridge and Hornby 2003 | Rothamsted little Knott field | Mercia | silty clay loam over clay-with-flints | untreated | 3rd | 14-Oct | 1992 | 1993 | March | 17.7 |
| Gutteridge and Hornby 2003 | Rothamsted little Knott field | Mercia | silty clay loam over clay-with-flints | untreated | 4th | 19-Oct | 1993 | 1994 | April | 13.8 |
| Gutteridge et al, 1986 | Rothamsted exp farm | Longbow | flinty clay loam soils | unkown | 3rd | 11-Oct | 1983 | 1984 | 16-Apr | 90.2 |
| Richard Gutteridge, Rothamsted | Rothamsted exp farm | Aquila | unkown | unkown | 3rd | 07-Sep | 1983 | 1984 | 16-May | 56 |
| Richard Gutteridge, Rothamsted | Rothamsted exp farm | Aquila | unkown | unkown | 3rd | 07-Oct | 1983 | 1984 | 16-May | 37 |
| Richard Gutteridge, Rothamsted | Rothamsted Stackyard | Oakley | unkown | unkown | 3rd | 09-Oct | 2008 | 2009 | 03-May | 73.4 |
| Richard Gutteridge, Rothamsted | Rothamsted West Barnfield | Oakley | unkown | unkown | 2nd | 03-Oct | 2008 | 2009 | 03-May | 35 |

**Table 6.2 Monsanto Ltd take-all survey**

| Harvest year | Treatment | County | Town | Nearest working weather station | Sowing date | Grid ref | Previous crop | Rotation | Soil type | Take-all GS31 |
|---|---|---|---|---|---|---|---|---|---|---|
| 2005 | Fungicide | Essex | Barnston | Andrewsfield | 12/10/2004 | TL 656 192 | Winter wheat | 2nd | Deep clay | 5.4 |
| 2006 | unknown | Essex | Barnston | Andrewsfield | 04/10/2005 | TL656192 | Winter wheat | 2nd | Deep clay | 8 |
| 2007 | Fungicide and PGR | Essex | Barnston | Andrewsfield | 13/10/2006 | TL658199 | Winter wheat | 2nd | D-clay | 3.3 |
| 2009 | Fungicide and PGR | Essex | Youngs End | Andrewsfield | 16/10/2008 | TL729203 | Winter wheat | 2nd | D-clay | 12 |
| 2010 | Fungicide and PGR | Essex | Bannister Green | Andrewsfield | 09/10/2009 | TL709213 | Winter wheat | 2nd | D-clay | 6 |
| 2005 | Fungicide | Lincolnshire | Baumber | Coningsby | 13/10/2004 | TF 233 723 | Winter wheat | 2nd | Light sand | 4 |
| 2006 | unknown | Lincolnshire | Hemingby | Coningsby | 19/10/2005 | TF250764 | Winter wheat | 2nd | Medium | 9.9 |
| 2007 | Fungicide and PGR | Lincolnshire | Baumber | Coningsby | 16/10/2006 | TF232734 | Winter wheat | 2nd | Medium | 13.7 |
| 2008 | Fungicide and PGR | Lincolnshire | Baumber | Coningsby | 16/10/2007 | TF235729 | Winter wheat | 2nd | Medium | 2 |
| 2009 | Fungicide and PGR | Lincolnshire | Horncastle | Coningsby | 21/10/2008 | TF252689 | Winter wheat | 2nd | Medium | 14 |
| 2010 | Fungicide and PGR | Lincolnshire | Great Sturton | Coningsby | 12/10/2009 | TF203769 | Winter wheat | 2nd | Medium | 8.8 |
| 2005 | Fungicide | Northumberland | Bowsden | Charterhall | 01/10/2004 | NU 002 415 | Winter wheat | 2nd | Deep clay | 11.9 |
| 2006 | unknown | Northumberland | Bowsden | Charterhall | 29/09/2005 | NT982404 | Winter wheat | 2nd | Deep clay | 9.4 |
| 2007 | Fungicide and PGR | Northumberland | Bowsden | Charterhall | 10/10/2006 | NT994422 | Winter wheat | 2nd | Medium | 1 |
| 2008 | Fungicide and PGR | Northumberland | Bowsden | Charterhall | 15/10/2007 | NU002417 | Winter wheat | 2nd | Medium | 2 |
| 2009 | Fungicide and PGR | Northumberland | Berwick-U-T | Charterhall | 06/10/2008 | NT995415 | Winter wheat | 2nd | Medium | 8 |
| 2005 | Fungicide | Suffolk | Stowmarket | Wattisham | 12/10/2004 | TL 973 597 | Winter wheat | 2nd | Light sand | 17 |
| 2006 | unknown | Suffolk | Gedding | Wattisham | 12/10/2005 | TL960590 | Winter wheat | 2nd | Medium | 2.4 |
| 2007 | Fungicide and PGR | Suffolk | Gedding | Wattisham | 03/10/2006 | TL981602 | Winter wheat | 2nd | Medium | 14.2 |
| 2008 | Fungicide and PGR | Suffolk | Bury St Edmunds | Wattisham | 14/10/2007 | TL976608 | Winter wheat | 2nd | Medium | 14 |
| 2009 | Fungicide and PGR | Suffolk | Woolpit | Wattisham | 16/10/2008 | TL988604 | Winter wheat | 2nd | Medium | 20 |
| 2010 | Fungicide and PGR | Suffolk | Woolpit | Wattisham | 14/10/2009 | TM006652 | Winter wheat | 2nd | D-clay | 3.1 |

available than data for summer take-all incidence. To identify crucial periods for take-all development, weather data was split into 28-day periods from sowing date, until spring assessment. A 28-day period was chosen as the allocated time period as it was the length of time for which the CE experiments were run.

### 6.3.2 CE experiments

Discussion of isolation and maintenance of *Ggt* stains is provided in Section 2.2.3.2. Three different strains of *Ggt* were used in this experiment from which agar plugs were placed in conical flasks containing sterile sand-maize meal medium. Flasks were kept moist and shaken twice a week for three weeks to encourage pathogen spread. When ready, sand-maize meal inoculum was mixed with pre-moistened vermiculite to obtain concentrations of 2 g 30 $mL^{-1}$ vermiculite, 0.2 g 30 $mL^{-1}$ vermiculite and 0.02 g 30 $mL^{-1}$ vermiculite. Falcon tubes were filled to 30 mL with vermiculite containing the different inoculum concentrations. Three sterilised Hereward seeds (see 2.2.1 for sterilisation method) were sown into the tubes and placed in the correct temperature regimes. Plants were grown in three separate controlled growth cabinets (Sanyo) with a 12 hr/12 hr day/night cycle, with a constant 80% relative humidity. Mean temperatures were chosen to simulate mean temperatures that are realistic of October indicated by the Rothamsted data; 8°C represented a cooler than average October, 10.5°C as an average and 13°C as a warmer than average October. Temperature regimes were set to minimum and maximum temperatures 3.5°C either side of the required mean temperature value. Cabinet temperature was programmed to ramp from the given minimum in the middle of the dark period to the given maximum in the middle of the light period with 2 hr increments between temperature changes. Temperatures are shown in Table 6.3.

To monitor growth of *Ggt* independently of the plant, plugs of inoculum were placed in Petri-dishes containing PDA in each temperature regime and growth was monitored over the experimental period. Plant roots were scored visually for percent roots infected and *Ggt* DNA levels were measured using qPCR. Roots for DNA extraction were freeze dried for two days before being ground using the procedure described in section 2.2.5. 800 µL of CTAB buffer was added to tubes which were vortexed then incubated for 30 mins at 65°C. 400 µL of Chloroform/Isoamylacohol (24:1) was added to tubes and vortexed for 15 secs

**Table 6.3 Mean, maximum and minimum temperatures of CERs for take-all experiments**

| Temp regime | Min temp | Max temp | Mean temp |
|---|---|---|---|
| 1 | 4.5°C | 11.5°C | 8°C |
| 2 | 7°C | 14°C | 10.5°C |
| 3 | 9.5°C | 16.5°C | 13°C |

centrifuged for 3 mins at 20000 x g. The upper phase of the samples was collected into a new tube and mixed with 400 µL isopropanol then incubated for 5 minutes at room temperature. After 15 mins at 20000 x g, the supernatant was discarded and the DNA pellet was washed with 300 µL 70 % ethanol by spinning for 3 mins at 20000 x g. The pellet was dried and re-suspended in TE buffer. DNA samples were adjusted to 10 ng/µL using a Picodrop® spectrophotometer. 5 µL of DNA was used in a 20 µL PCR reaction with 0.4 µL of *Ggt* primers (2a and 2b in Table 2.2) at 10 µM, 10 µL of SYBR® Green JumpStart™ Taq Readymix (Sigma-Aldrich) and 4.2 µL of dH2O. To determine *Ggt* levels in each root, a PCR reaction containing a serial dilution of *Ggt* DNA at a known concentration was run on the same PCR plate. 5 µL of *Ggt* DNA (extracted from one of the strains used in this experiment using the procedure outlined in 2.2.3.2) was used in the PCR reaction outlined above. Cycling conditions were according to section 2.2.7. A standard curve was calculated from the results of the *Ggt* serial dilution which was used to calculate the amount of *Ggt* in each root sample (Figure 6.1).

For experiments to monitor the initial interaction between wheat and *Ggt*, Hereward seeds were sterilised according to section 2.2 and placed at one end of a 9 x 9 cm square Petri-dish containing 1% water agar which was covered by sterilised filter paper. Plugs of the same three *Ggt* isolates described above were transferred onto the same plate at random locations to represent *Ggt* inoculum in the soil. Plates were wrapped in aluminium foil with an opening at one end, which exposed seeds to light, and placed in one of two CE cabinets (Schneider) at a 45$^{o}$ angle. CE's were programmed with a 12 hr/12 hr day/night cycle, the first set to a constant 8°C and the second to a constant 13°C (being the lowest and highest October mean temperatures observed in the Rothamsted data). Plates were monitored over a 28 day period during which various measurements at different time points were

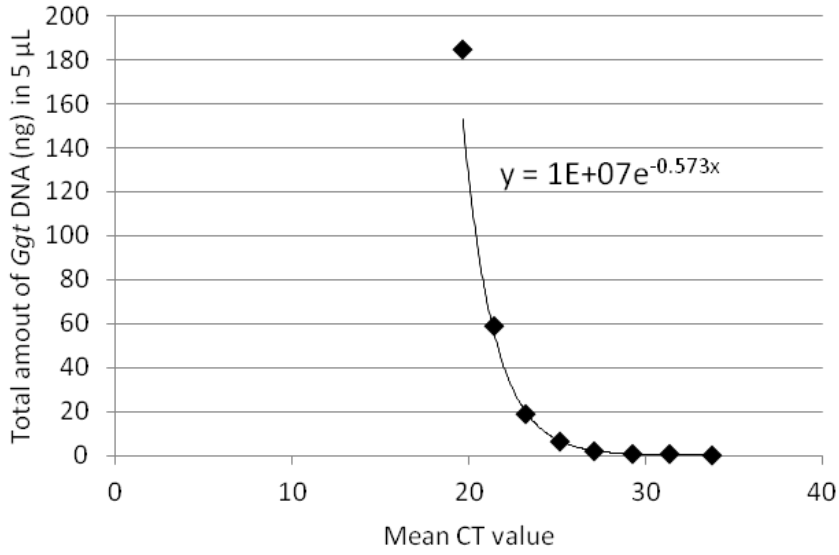



**Figure 6.1 Standard curve to determine amount of *Ggt* DNA in total DNA from Ct values generated by qPCR reaction.** A serial dilution of a known concentration of *Ggt* DNA was generated and run along side samples in a qPCR reaction to determine the amount of *Ggt* in each root sample.

recorded (Figure 6.2). During the monitoring, plates were given an additional 1 mL of dH2O on a weekly basis. As in the previous CE experiment, independent plugs of *Ggt* inoculum were placed in Petri-dishes containing PDA in each temperature regime and growth was monitored over the experimental period.

### 6.3.3 Statistical analysis

Historical data were analysed using the linear regression analysis in Microsoft Office Excel package. Data from CE experiments were analysed with general linear regression using the statistical package Genstat for Windows, release 12 (VSN international, Hemel Hempstead, UK). ANOVA tables can be found in the appendix. For CE experiment 15 or more individual plant replicates were used to calculate means and standard error unless otherwise specified. Locations of tubes/plates were changed throughout the experimental period as a means of randomisation.

## 6.4 Results

### 6.4.1 Soil temperature is strongly related to air temperature in soil type at the Rothamsted site

Daily mean air temperature was compared to daily mean soil temperature at Rothamsted over a 10 year period between 2000 and 2010 using the data supplied by e-RA. Temperatures were taken at soil depths of 10 cm and 30 cm although *Ggt* inoculum is not thought to be the cause of significant infection at depths greater than 10 cm (Cotterill 1988). Results showed that mean air temperature is extremely significantly correlated with soil temperature at both 10 cm (Appendix, Table A1, $P < 0.001$) and 30 cm (Appendix, Table A2, $P < 0.001$, Figure 6.3). The maximum temperature difference between air temperature and soil temperature in the 10 year period was 5.65°C and the average difference between soil and air temperature was 0.16°C.

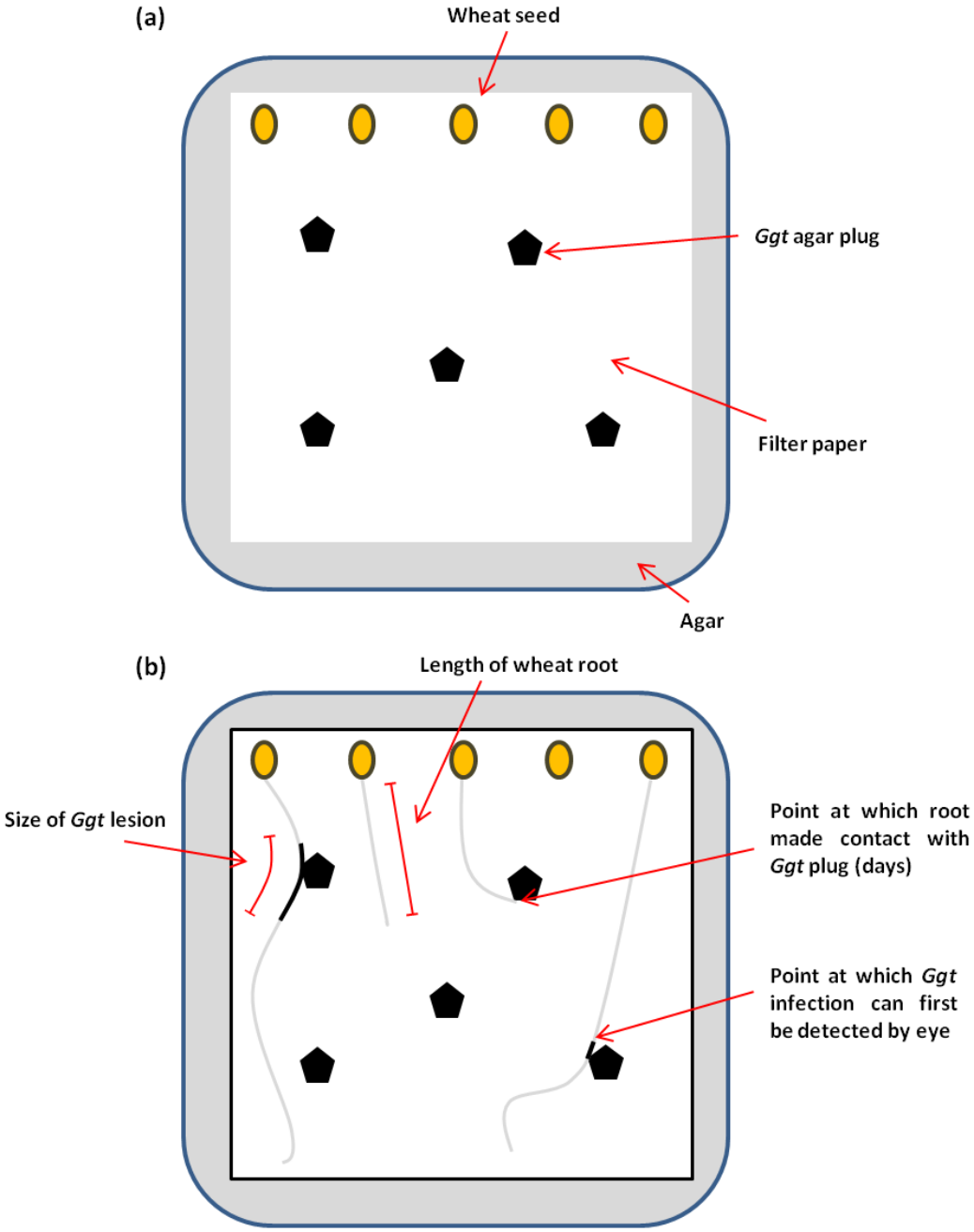



**Figure 6.2 Diagram of experimental set up to measure the dynamics of the wheat-*Ggt* interaction at early stages. (a)** Initial set up of seeds and Ggt plugs **(b)** Indication of measurements taken over the 28 day period.

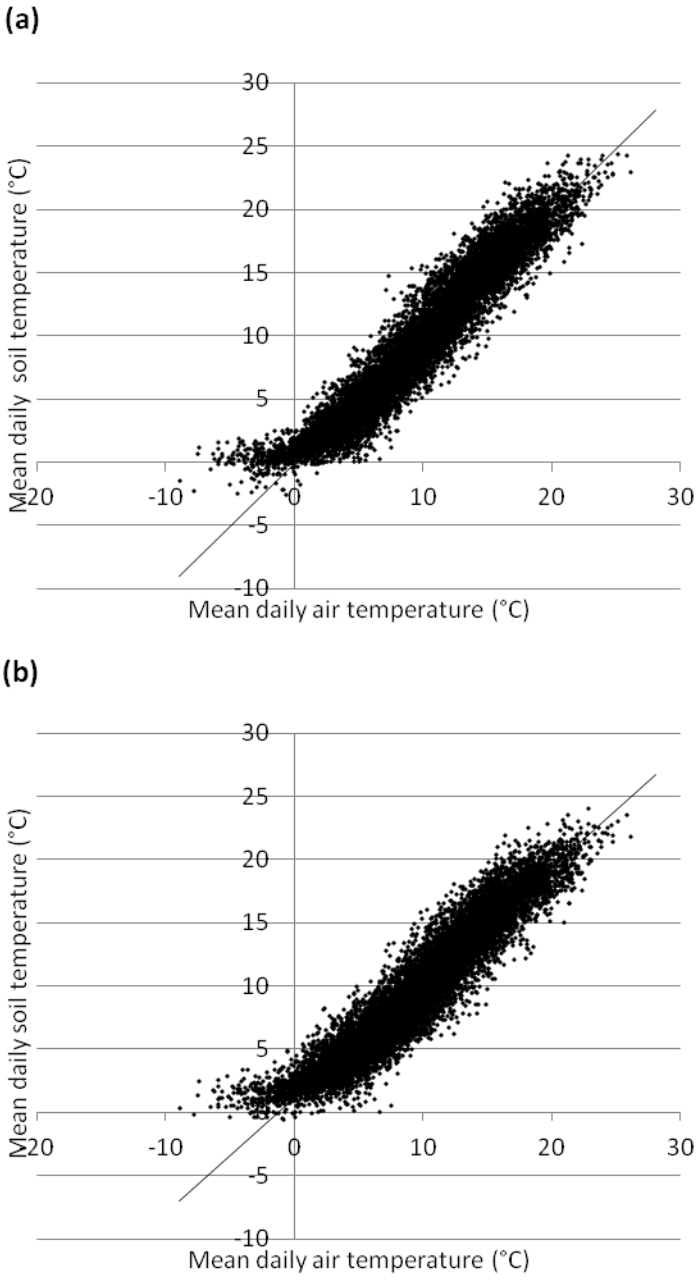



**Figure 6.3 Soil temperature is strongly influenced by air temperature in soil type at Rothamsted site.** Relationship between mean daily air temperature and mean daily soil temperature over a 10 year period between 2000 and 2010 at soil depth of **(a)** 10 cm, ($P < 0.001$) and **(b)** 30 cm ($P < 0.001$). Data were obtained from e-RA and analysed using a multiple regression analysis.

### 6.4.2 Mean air temperature during early post sowing period affects spring take-all levels in 3rd and 4th wheat rotations sown at Rothamsted

Rothamsted historical disease records extracted from various publications over a 30 year period were compared with local climate data to look for relationships between temperature, rainfall and spring disease levels. To identify important climatic periods for take-all development, mean air temperatures and rainfall were split into 28-day periods between sowing dates and the first take-all assessment. Results showed no significant correlations between spring take-all levels in 2nd wheat and mean temperature or total rainfall in any of the 28 day periods (Figure 6.4b). For 3rd wheat, there was a significant positive correlation between mean temperature in the first 28 day period post sowing and spring take-all disease levels (Appendix, Table A3, *P = 0.03*, Figure 6.4a,b). Mean temperature from the subsequent 28 day periods did not show a significant correlation with 3rd wheat spring take-all levels (Figure 6.4a). For 4th wheat, the positive correlation between average temperature in the 28 day period post sowing and spring take-all disease levels was even more significant (Appendix, Table A4, *P < 0.01,* Figure 6.4a,b). The next 28 day period also had a significant positive correlation (Appendix, Table A5, *P = 0.03*), although no mean temperature of subsequent 28 day periods showed a significant correlation with spring take-all levels (Figure 6.4b). A negative correlation was observed between total rainfall and spring take-all levels in the 7th 28 day period after sowing in 2nd wheat which would be around the time plants were assessed in the spring (*P < 0.01*, Figure 6.4a). Another negative correlation between total rainfall and spring take-all levels was observed in the 3rd 28 day period after sowing of 4th wheat, which would fall in the middle of winter (Appendix, Table A6, *P = 0.03*, Figure 6.4a). Length of time that wheat was in the ground before assessment was not significantly correlated with spring take-all levels (Table 6.4).

### 6.4.3 An effect of initial temperatures on spring take-all levels is seen in 2nd and 3rd wheat rotations in long term experiment sown at Rosemaund, Herefordshire

Data from a 6 year experiment by Spink *et al* (2004) was analysed in the same way as the Rothamsted data in section 6.4.2. Results showed no significant correlations between spring take-all levels in 1st wheat and temperature or rainfall in any of the 28 day periods

**(a)**

| Rotation | Period of time after sowing (days) | Mean temperature | | Total rainfall | |
|---|---|---|---|---|---|
| | | *P* | $r^2$ | *P* | $r^2$ |
| 2nd | 0-28 | 0.77 | 0.02 | 0.24 | 0.32 |
| 2nd | 29-56 | 0.37 | 0.20 | 0.36 | 0.21 |
| 2nd | 57-85 | 0.56 | 0.09 | 0.54 | 0.10 |
| 2nd | 86-114 | 0.69 | 0.04 | 0.68 | 0.05 |
| 2nd | 115-143 | 0.38 | 0.20 | 0.56 | 0.09 |
| 2nd | 144-172 | 0.10 | 0.60 | 0.92 | 0.01 |
| 2nd | 173-201 | 0.39 | 0.18 | <0.01* | 0.82 |
| 3rd | 0-28 | 0.03* | 0.44 | 0.93 | 0.00 |
| 3rd | 29-56 | 0.09 | 0.29 | 0.51 | 0.05 |
| 3rd | 57-85 | 0.17 | 0.20 | 0.74 | 0.01 |
| 3rd | 86-114 | 0.59 | 0.03 | 0.41 | 0.08 |
| 3rd | 115-143 | 0.28 | 0.13 | 0.23 | 0.16 |
| 3rd | 144-172 | 0.27 | 0.13 | 0.98 | 0.00 |
| 3rd | 173-201 | 0.48 | 0.06 | 0.20 | 0.18 |
| 4th | 0-28 | <0.01* | 0.91 | 0.50 | 0.12 |
| 4th | 29-56 | 0.02* | 0.74 | 0.50 | 0.12 |
| 4th | 57-85 | 0.06 | 0.61 | 0.03* | 0.73 |
| 4th | 86-114 | 0.63 | 0.06 | 0.63 | 0.02 |
| 4th | 115-143 | 0.28 | 0.28 | 0.19 | 0.38 |
| 4th | 144-172 | 0.68 | 0.05 | 0.49 | 0.13 |
| 4th | 173-201 | 0.56 | 0.09 | 0.47 | 0.14 |

**(b)**

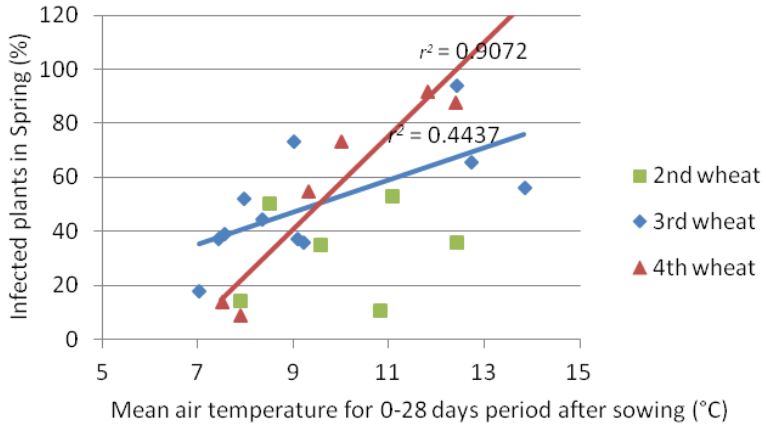



**Figure 6.4 Mean temperature during early post sowing period affects spring take-all levels in 3rd and 4th wheat rotations sown at Rothamsted. (a)** Correlation significance (*P*) and coefficient ($r^2$) for associations between mean temperature (°C) and spring take-all levels (% plants infected) over several years. Data are split into successive 28 day periods post sowing and analysed using a regression analysis. Each $r^2$ and *P* value in the table is calculated using data from six independent data points for 2nd wheat, eleven independent data points for 3rd wheat and six independent data points for 4th wheat. Significant differences are indicated with an asterisk **(b)** Correlations for associations between mean temperature in 0-28 days post sowing and spring take-all levels from Figure 6.3a.

| Wheat rotation | Time between sowing and assessment (days) | |
|---|---|---|
| | *P* | $r^2$ |
| 2nd | 0.20 | 0.64 |
| 3rd | 0.71 | 0.02 |
| 4th | 0.64 | 0.13 |
| All | 0.34 | 0.06 |

**Table 6.4 Time between sowing and assessment date does not have an effect on spring take-all levels at Rothamsted.** Correlation significance (*P*) and coefficient ($r^2$) for associations between time from sowing till assessment date (days) and spring take-all levels (% plants in infected) from Rothamsted data. Data were analysed using a regression analysis with different wheat rotations and with all data points from every wheat rotation.

post sowing (Table 6.5). For 2nd and 3rd wheat a significant positive correlation was only apparent between average temperature in the first 28 day period post sowing and spring take-all disease levels (2nd wheat, Appendix, Table A7, *P = 0.04*, 3rd wheat, Appendix, Table A8, *P = 0.01*, Table 6.5). The trend was stronger and more significant in 3rd wheat than 2nd wheat. No correlation was observed between total rainfall and spring take-all levels in any of the wheat crop rotations across all the 28 day periods (Table 6.5).

### 6.4.4 No effect of initial temperature was seen on 2nd wheats from various UK locations

The relationship between temperature and rainfall and spring take-all levels was further investigated using data from various Monsanto Ltd/NIAB trials, grown in different UK regions. All wheat was in a 2nd rotation. Data showed that mean temperatures in the first 28 day period post-sowing had no effect on spring take-all levels at any of the locations, as for the Rothamsted result for second wheats (Table 6.6). At one location (Northumberland), a weak significant positive correlation was observed between rainfall in this same period and spring take-all levels (Appendix, Table A9, *P = 0.05*, Table 6.6). No other location showed that rainfall levels had any effect on spring take-all levels.

### 6.4.5 Mean temperature has the greatest effect on take-all at the highest *Ggt* concentrations

Analysis of historical data suggested that initial temperature might be an important factor influencing spring take-all levels. To confirm whether the relationship between initial growth temperatures and take all infection could be seen under CE conditions, and to test whether different concentrations of *Ggt* inoculum were differentially affected by temperature, inoculum was added to the growth media in 10 fold dilutions, with plants grown at a range of temperatures that were realistic to UK sowing temperatures. Infection rates and *Ggt* concentrations were fitted to the generalised linear model temperature and inoculum concentration. Results indicated that there was an effect of both temperature and concentration on both percentage of roots infected and *Ggt* DNA levels but there was no interaction between the two variables. At the lowest *Ggt* concentration (0.02g/tube), the number of roots infected were significantly different between all three temperature regimes (*P < 0.01*, Figure 6.5a). Plant root *Ggt* levels followed a similar trend at this concentration although there was no significant difference between plants grown at mean

| Rotation | Period of time after sowing (days) | Mean temperature | | Total rainfall | |
|---|---|---|---|---|---|
| | | *P* | $r^2$ | *P* | $r^2$ |
| 1st | 0-28 | 0.43 | 0.22 | 0.36 | 0.28 |
| 1st | 29-56 | 0.18 | 0.51 | 0.84 | 0.02 |
| 1st | 57-85 | 0.46 | 0.19 | 0.13 | 0.59 |
| 1st | 86-114 | 0.52 | 0.15 | 0.31 | 0.33 |
| 1st | 115-143 | 0.78 | 0.03 | 0.82 | 0.02 |
| 1st | 144-172 | 0.25 | 0.40 | 0.85 | 0.01 |
| 1st | 173-201 | 0.35 | 0.29 | 0.55 | 0.13 |
| 2nd | 0-28 | 0.04* | 0.80 | 0.82 | 0.02 |
| 2nd | 29-56 | 0.98 | 0.00 | 0.22 | 0.45 |
| 2nd | 57-85 | 0.46 | 0.19 | 0.46 | 0.19 |
| 2nd | 86-114 | 0.51 | 0.16 | 0.13 | 0.58 |
| 2nd | 115-143 | 0.19 | 0.49 | 0.19 | 0.48 |
| 2nd | 144-172 | 0.71 | 0.05 | 0.46 | 0.19 |
| 2nd | 173-201 | 0.87 | 0.01 | 0.59 | 0.11 |
| 3rd | 0-28 | 0.01* | 0.97 | 0.50 | 0.25 |
| 3rd | 29-56 | 0.89 | 0.01 | 0.09 | 0.83 |
| 3rd | 57-85 | 0.37 | 0.40 | 0.56 | 0.19 |
| 3rd | 86-114 | 0.82 | 0.03 | 0.37 | 0.40 |
| 3rd | 115-143 | 0.07 | 0.87 | 0.31 | 0.47 |
| 3rd | 144-172 | 0.90 | 0.01 | 0.52 | 0.23 |
| 3rd | 173-201 | 0.85 | 0.02 | 0.61 | 0.15 |

**Table 6.5 Mean temperature during early post sowing period affects spring take-all levels in 2nd and 3rd wheat rotations sown at Rosemaund, Herefordshire.** Correlation significance (*P*) and coefficient ($r^2$) for associations between mean temperature (°C) during successive 28 day periods post sowing and spring take-all levels (index scores) collected over several years. Data were obtained from Spink et al (2004) and analysed using a regression analysis. Each $r^2$ and *P* value in the table is calculated using data from five different years for 1st and 2nd wheat and four years for 3rd wheat.

| Location | Mean temperature | | Total rainfall | |
|---|---|---|---|---|
| | *P* | $r^2$ | *P* | $r^2$ |
| Essex | 0.59 | 0.11 | 0.42 | 0.23 |
| Lincolnshire | 0.74 | 0.03 | 0.63 | 0.07 |
| Northumberland | 0.75 | 0.04 | 0.05* | 0.76 |
| Suffolk | 0.18 | 0.40 | 0.74 | 0.03 |

**Table 6.6 Mean temperature during early post sowing period does not affect spring take-all levels in 2nd wheat rotations sown at various UK locations.** Correlation significance (*P*) and coefficient ($r^2$) for associations between mean temperature (°C) and total rainfall (mm) during the 0-28 day period post sowing and 2nd wheat spring take-all levels (index scores) collected over several years at various UK locations. Data were obtained from Monsanto Ltd and analysed using a regression analysis. Each $r^2$ and *P* value in the table is calculated with data from six different years for Suffolk and Essex and five different years for Lincolnshire and Northumberland.

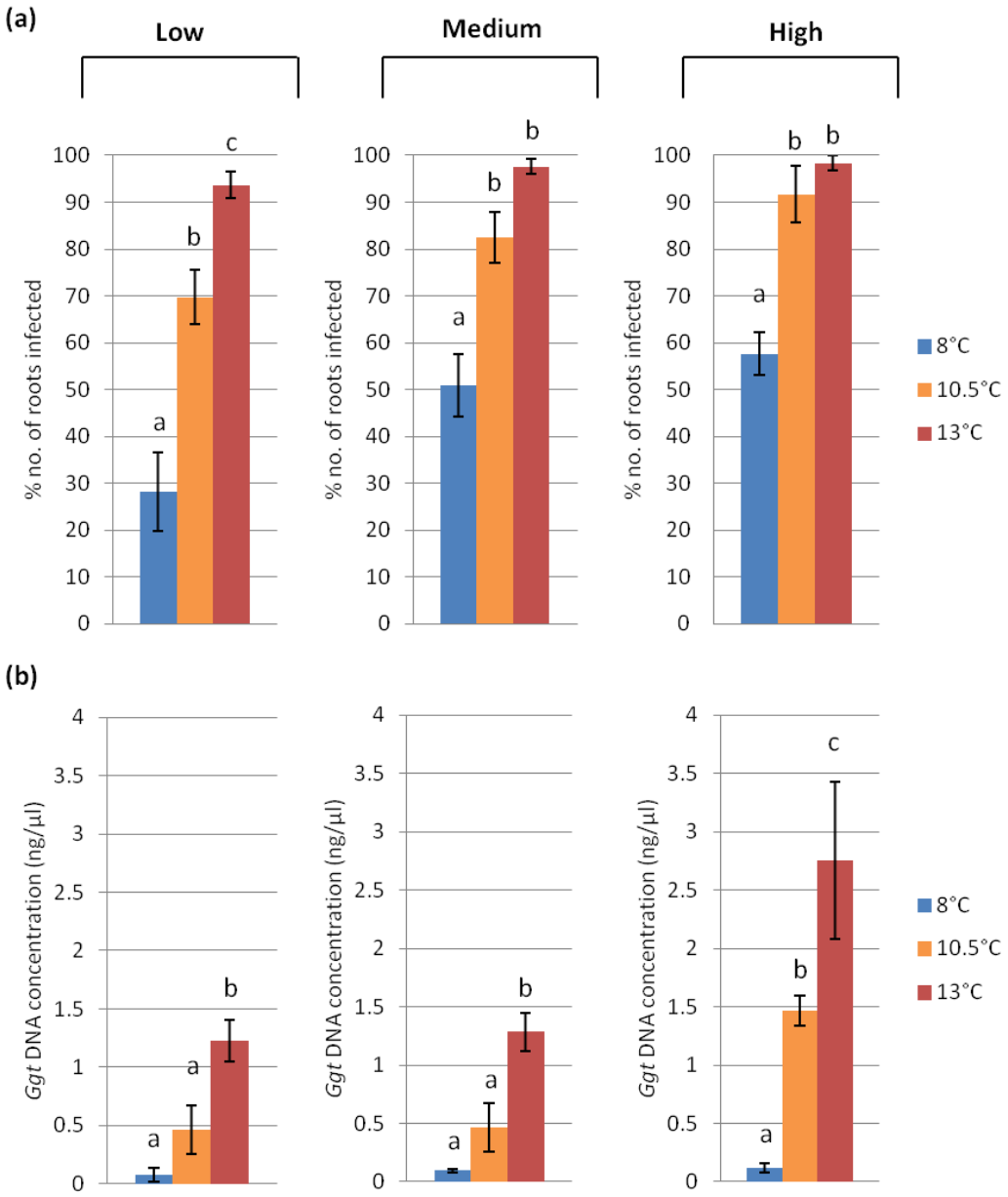



**Figure 6.5 Effect of temperature on take-all disease using different concentrations of *Ggt*.** Plants were grown at different temperatures in different concentrations of *Ggt* inoculum and assessed for take-all infection after 4 weeks. Concentrations of *Ggt* inoculum are indicated above by low (0.02g 30 $mL^{-1}$ vermiculite), medium (0.2g 30 $mL^{-1}$ vermiculite) and high (2g 30 $mL^{-1}$ vermiculite) labels above. **(a)** Bars represent mean no. of roots infected (± 1 SE) from 15 biological replicates for which data were analysed with the generalised linear model. The model showed that there was an effect of temperature and concentration on % roots infected but that there was no interaction between the two variables. Different letters indicate a statistically significant difference between temperature treatments ($P < 0.05$). **(b)** Bars represent mean DNA levels (± 1 SE) from 5 biological reps containing three plant roots which was calculated using a standard curve of *Ggt* inoculum at a known concentration. The model showed that there was an effect of temperature and concentration on *Ggt* DNA levels but that there was no interaction between the two variables. Different letters indicate a statistically significant difference between temperature treatments ($P < 0.05$).

temperatures of 8°C and 10.5°C (Figure 6.5b). At intermediate *Ggt* concentrations (0.2g), the number of roots infected was significantly different between plants grown at 8°C and 10.5°C (*P < 0.001*) but there was no significant difference between plants grown at 10.5°C and 13°C, presumably because disease saturation had occurred in plants grown at the highest temperature (Figure 6.5a). Again, plant root *Ggt* levels followed a similar trend to number of roots infected and similar to the lower *Ggt* concentration, there was no significant difference in *Ggt* levels between plants grown at a mean temperature of 8°C and 10.5°C (Figure 6.5b). High *Ggt* concentrations (2g) gave a parallel result to intermediate concentrations, in that number of roots infected was significantly different between plants grown at 8°C and 10.5°C (*P < 0.001*) and once more there was no significant difference between plants grown at 10.5°C and 13°C (Figure 6.5a). At this concentration, plants grown in all three temperature regimes had significantly different *Ggt* levels in plant roots (Figure 6.5b). Low and intermediate *Ggt* inoculum concentrations produced root *Ggt* level that differed approximately 5 fold between plants grown at 8°C and 10.5°C, whereas the highest *Ggt* inoculum concentration had a fold change of 12 between plants grown at 8°C and 10.5°C. Likewise, fold change of *Ggt* levels was more extreme in the highest inoculum concentration between plants grown at 8°C and 13°C (Figure 6.5b). *Ggt* isolateswere also grown independently on agar plates to monitor external growth of pathogen. All three strains grew quickest at 13°C, slowest at 8°C and at an intermediate rate at 10.5°C (Figure 6.6).

### 6.4.6 Plant root growth rate and *Ggt* infection spread is more extreme at 13°C compared to 8°C

To determine how temperature affects the initial wheat-*Ggt* interaction, an experiment to simulate the 28 day period post sowing under two different temperatures was performed. Plant root growth rate was three times faster at 13°C than at 8°C and roots took an average of 9.45 days to come into contact with a *Ggt* inoculum source at the lower temperature compared to an average of 4.47 at the higher temperature (Figure 6.7a,b,c). Despite the roots only taking twice as long to make contact with the *Ggt* inoculum at 8°C compared to 13°C, on average it took *Ggt* almost five times longer to infect plant cells at the lower temperature the difference being an average of 9.68 days at 8°C and an average of only 2.07 at 13°C (Figure 6.7a,c,d). *Ggt* induced lesions spread through the plant tissue kept at

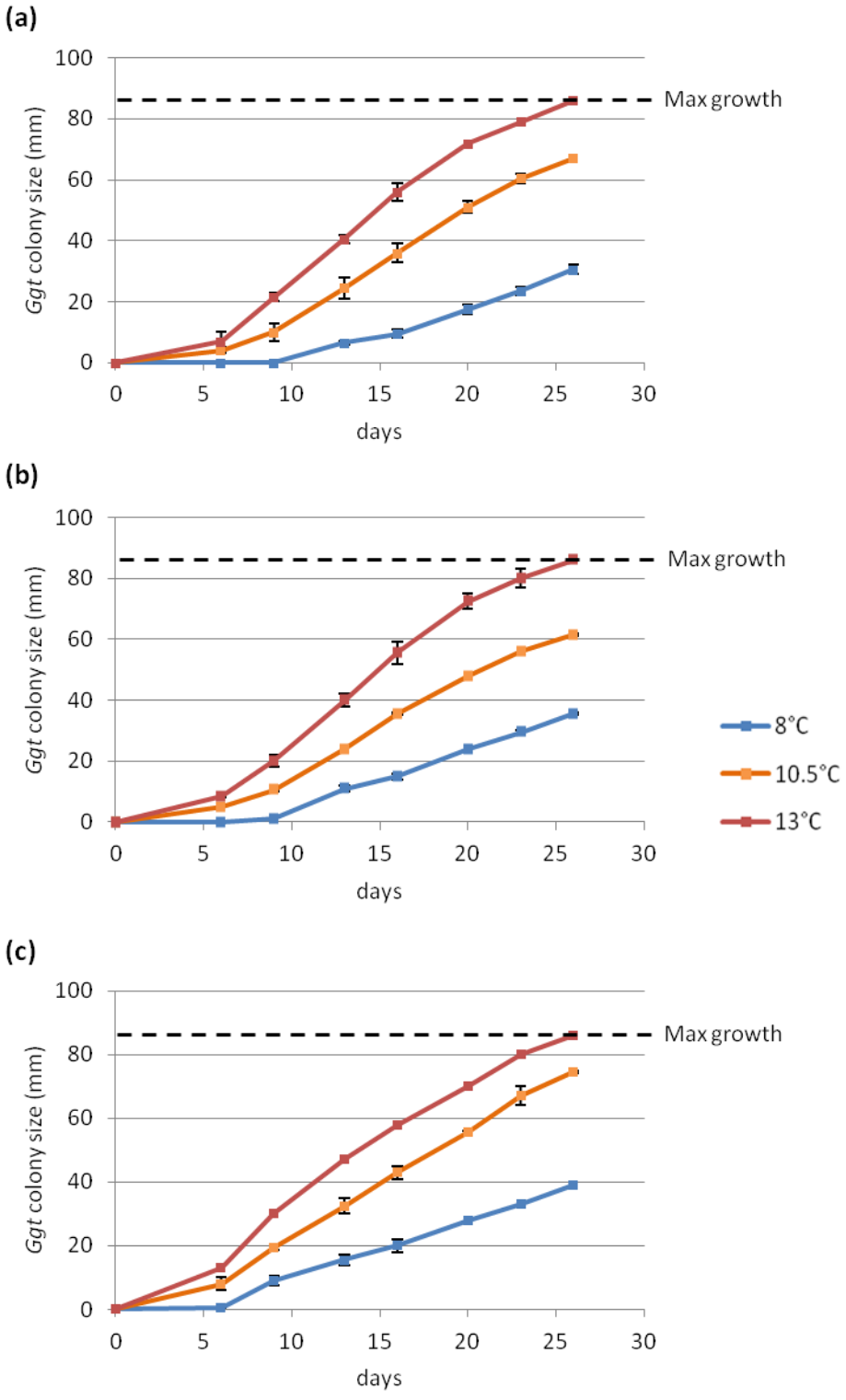
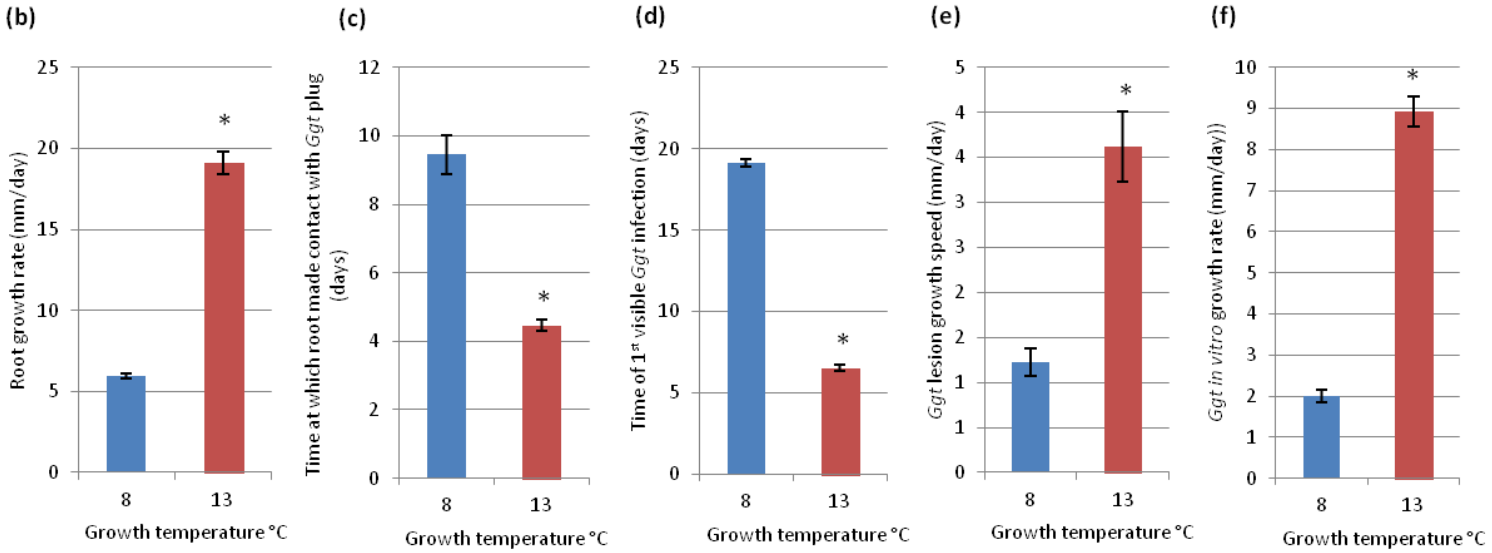



**Figure 6.6 Effect of temperature on *Ggt* growth.** To monitor external *Ggt* growth different *Ggt* strains were grown at the same temperatures and time as infected plants in Figure 5.3. Graphs with different letters are the three strains used in the CER experiment. Data points represent mean size of fungal colony from three plate replicates (± 1 SE). The black dotted line represents max possible growth of fungus due to plate size restrictions.

**Figure 6.7 (overleaf) Comparison of the *Ggt* infection process between plants grown at 8°C and plants grown at 13°C.** Wheat seeds of cultivar Hereward were germinated on filter paper in petri-dishes containing water agar with *Ggt* plugs at various locations. Plates were incubated in CE cabinets at temperatures if 8°C or 13°C and observations regarding the plant-pathogen interaction were recorded over a 28 day period. *Ggt* plugs were also placed in separate petri-dishes containing PDA to monitor *in vitro* growth. **(a)** Summary of measurements taken, columns show mean values (± 1 SE) for each isolate and total means (± 1 SE) for all isolates. Significant differences between total means at 8°C and 13°C are indicated by different letters (*P < 001*). Means for all isolates are also shown graphically:- **(b)** comparison of root growth rate, **(c)** comparison of the time at which root made contact with the *Ggt* plug, **(d)** comparison of the time at which the first visible *Ggt* infection was seen, **(e)** comparison of the speed at which the Ggt lesion spread through the plant tissue and **(f)** comparison of the speed at which *in vitro Ggt* colonies grew on plates. Significant differences between treatments are indicated by an asterisk.

(a)

| Temperature (°C) | *Ggt* isolate | Root growth rate (mm/day) | Time at which root made contact with *Ggt* plug (days) | Time of 1st visible *Ggt* infection (days) | *Ggt* lesion growth speed (mm/day) | *Ggt in vitro* growth rate (mm/day) |
|---|---|---|---|---|---|---|
| 8 | a | - | - | 20.13 ± 1.27 | 1.11 ± 0.27 | 2.13 ± 0.13 |
| | b | - | - | 17.25 ± 0.80 | 1.43 ± 0.24 | 2.00 ± 0.25 |
| | c | - | - | 20.14 ± 1.01 | 0.96 ± 0.31 | 1.88 ± 0.13 |
| | all | 5.96 ± 0.14 **a** | 9.45 ± 0.56 **a** | 19.13 ± 0.65 **a** | 1.22 ± 0.15 **a** | 2.00 ± 0.16 **a** |
| 13 | a | - | - | 6.17 ± 0.17 | 3.39 ± 0.72 | 7.58 ± 0.17 |
| | b | - | - | 7.20 ± 0.49 | 4.50 ± 0.35 | 9.17 ± 0.22 |
| | c | - | - | 6.25 ± 0.25 | 3.08 ± 0.72 | 10.00 ± 0.29 |
| | all | 19.10 ± 0.70 **b** | 4.47 ± 0.16 **b** | 6.53 ± 0.22 **b** | 3.62 ± 0.39 **b** | 8.92 ± 0.37 **b** |



13°C approximately three times the rate at which infection spread in plants at 8°C (Figure 6.7a,e). The increased growth rate at 13°C was also observed in the *in vitro Ggt* colonies, but the increased rate of growth at 13°C was slightly higher being approximately four fold on average (Figure 6.7a,f).

### 6.4.7 Current and future mean temperatures during typical UK sowing time for Hertfordshire and Herefordshire

Historical data from the Met Office reveals that current mean temperatures during October (sowing time) in Hertfordshire and Herefordshire are similar, being about 10.5 °C (Figure 6.8). *UKCP09* climate predictions suggest that temperature increases will be greater in Hertfordshire compared to Herefordshire using a medium emission scenario. In Herefordshire, the highest recent October temperatures are projected to be commonplace in 2080 whereas in Hertfordshire current extreme October temperatures are projected to be commonplace in 2050. By 2080, mean October temperatures in Hertfordshire will be almost 1°C warmer than the most extreme October temperatures in recent years (Figure 6.8).

## 6.5 Discussion

Results show a relationship between initial post sowing temperatures and spring take-all levels in winter wheat, considering a positive correlation was observed between both 3$^{rd}$ and 4$^{th}$ wheat rotations grown at Rothamsted, and both 2$^{nd}$ and 3$^{rd}$ wheat rotations grown at Rosemaund, Herefordshire. This result is supported by findings of Smiley *et al* (2009) which indicate that mean temperature for 21 days post planting shows a positive correlation with spring take-all levels in the US. Findings by Lucas *et al* (1998) also show that temperatures in the initial period post sowing are important. Additional support comes from CE experiments which showed that plants grown at a higher mean temperature had increased take-all symptoms 28 dpi. However no relationship was seen in 1$^{st}$ and 2$^{nd}$ wheat rotations at Rothamsted, 1$^{st}$ wheat rotations at Rosemaund and 2$^{nd}$ wheat at other various UK locations. *Ggt* inoculum in the field is not uniformly distributed and is built up year on year by successive wheat crop rotations (Colbach *et al.*, 1997). Results

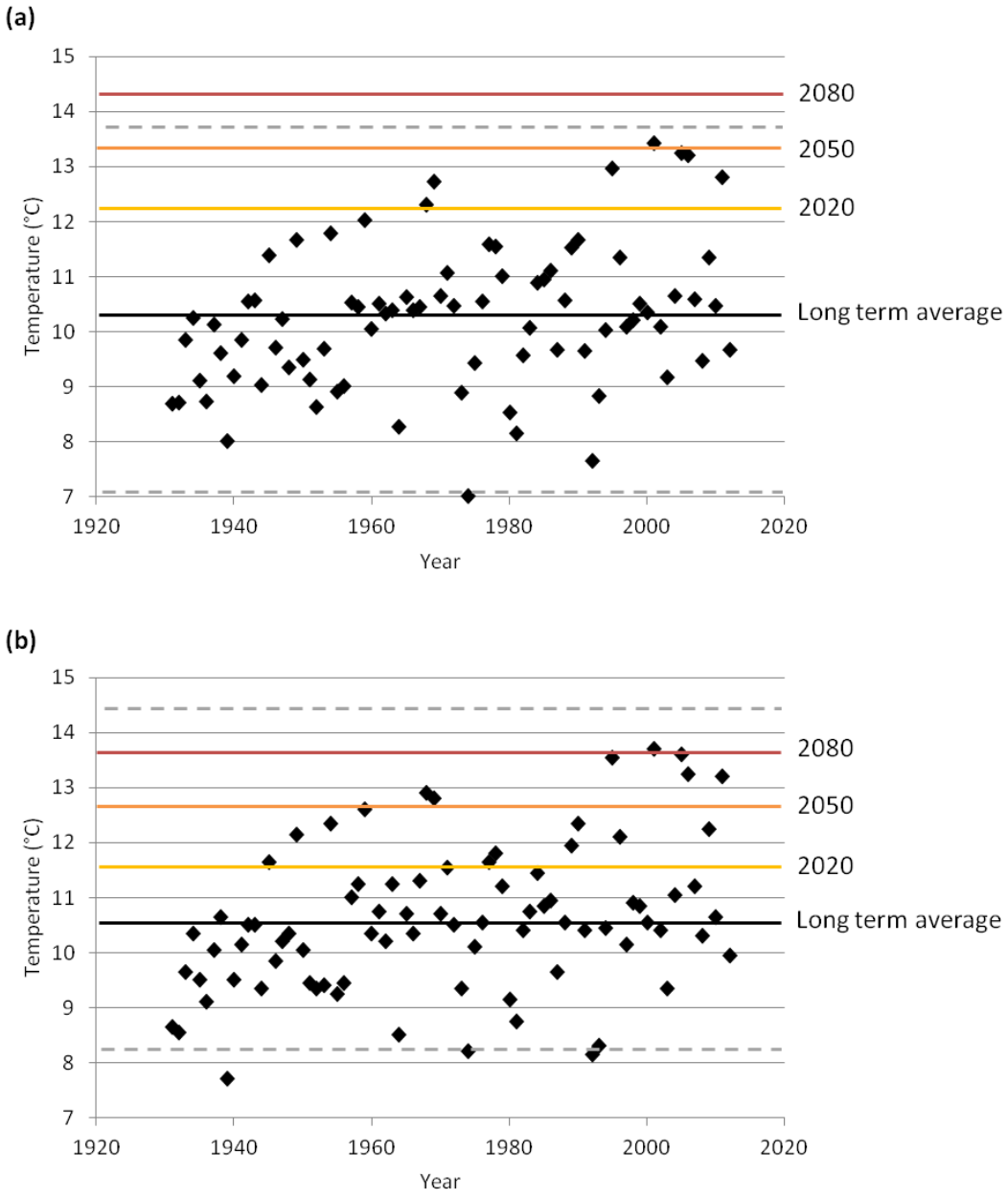



**Figure 6.8 Average October temperatures are predicted to increase at different rates in different locations. (a)** Mean October temperatures in Rothamsted. Long term average has been calculated from the 80 year period of data presented on the graph. Predictions for mean October temperature in 2020, 2050 and 2080 are based on 30 year averages generated from *UKCP09* using a medium emissions scenario. Grey dotted lines represent the range of temperatures that were observed at the Rothamsted site during the 0-28 day period post sowing and used in the data analysis **(b)** As above for mean October temperatures in Ross on Wye.

presented in this chapter suggest that the relationship between autumn sowing temperature and spring take-all levels can only be seen once inoculum concentrations have reached a particular threshold, due to the effect not being apparent until later wheat rotations. This is supported by CE data in that the effect of temperature on take-all levels was strongest at the highest concentration compared to the low and intermediate concentration, which were not significantly different. It is possible that the relationship between mean temperature and spring take-all levels was seen earlier at the Rosemaund site than the Rothamsted site due to soil *Ggt* levels being naturally higher at the Rosemaund. Generally a relationship between temperature and take-all is not seen in 2nd wheat rotations suggesting the experiment at Rosemaund may be an exception. This exception could be due to a number of factors including soil type, other climatic factors or agricultural practises. It is possible that the Monsanto/NIAB data may not have shown any correlations between initial temperatures and spring take-all because the relationship is not generally seen in 2nd wheat rotations. However the trials were grown at various locations within a 20 miles radius creating many interfering factors influencing results such as different soil types and local climate discrepancies.

Given that post sowing mean temperatures impact on spring take-all levels, this knowledge could be used in take-all preventative agricultural management. For example, identification of influential climatic factors will aid in more accurate modelling of disease epidemiology. What's more, if autumn and winter temperatures are predicted to be mild for the year in question, growers may benefit from later sowing or not risking sowing a 2nd or 3rd wheat rotation. Traditionally in the Hereford area, a 2nd wheat rotation would occasionally be grown but not a 3rd due to high risk of take-all (Spink *et al.*, 2004). Future conditions may eliminate the possibility of growing even a 2nd wheat in this location. Many farmers will risk a 2nd or 3rd wheat (Hammond-Kosack, 2011; Jones, 2009), presumably due to increased profit potential if the risk pays off. In the later part of this century the risk may not be worth taking. Climate predictions provided by *UKCP09* suggest that some areas of the UK will warm more quickly than other areas (Murphy *et al.*, 2009). Results indicate that mean temperatures will rise more quickly in the East of England (Figure 6.8). If the East warms more quickly than the West, Eastern areas may face earlier problems with wheat rotations which is especially unfortunate due to its current status as the main wheat growing region.

The results suggest that the effect of increasing temperatures from climate change is specific to sites. Therefore to assess impact of climate change on take-all using UKCP09,

access to more regional data would need to be obtained. Predictions by Evans *et al* (2008; in a study regarding how climate change would influence infection by phoma stem canker on oil seed rape) were developed using a phoma stem canker model developed by previous findings from various studies (Sun *et al*., 2000; West *et al*., 2001). The model was validated prior to combining with UKCIP02 outputs, suggesting that a similar model for take all would need to be developed before output could be combined with UKCP09. The high resolution of UKCP09 would make this possible, but would require long term monitoring of take-all at an equally high resolution and would have to take into account soil type. UKCP09 also only has air temperature as an output whereas *Ggt* is also soil borne, but the highly significant correlation between air and soil temperature enhances the value of UKCP09 for making climate change predictions at a below ground level.

Colbach *et al* (1997) argue that early sowing increases disease via the primary infection cycle. Results from analysing the Rothamsted data do not support this hypothesis as there was no relationship between length of time that wheat was in the ground and percentage of roots infected in the spring (Table 6.4). Early autumn sowings will normally have warmer temperatures compared to those sown later. The reason why early sowings are correlated with increased take-all could be due to higher temperatures independently of the time that wheat is in the ground. Results indicate that temperature only appears to be important in the initial period after sowing and that temperature does not subsequently show a relationship with spring take-all levels. The only exception to this is the 2$^{nd}$ 28 day period from the 4$^{th}$ wheat grown at Rothamsted. This suggests that temperature has a stronger effect on primary, rather than secondary, take-all infection. Perhaps the *Ggt* ability to reach the plant is more strongly affected by temperature than the ability of the fungus to spread through the plant. However, there is no evidence that *Ggt* can grow in the soil as levels tend to decrease overtime (Bithell *et al*., 2009). Nevertheless, it is interesting that *in vitro Ggt* growth rate shows a bigger difference between 8°C and 13°C than growth of the fungus in the plant.

In three months post harvest*, Ggt* inoculum can decline by between 70% and 25% depending on the field (Bithell *et al*., 2009). Results here showed that root growth was slower when temperatures were cooler so another hypothesis is that increase in temperature increases root growth, so roots come into contact with more *Ggt* inoculum before it is broken down in the soil. In addition, *Ggt* was strikingly slow at infecting root tissue at 8°C compared to 13°C, perhaps signifying that the combination of delayed contact

of wheat roots with a *Ggt* inoculum source, and an even more delayed infection time, contributes to decreased primary infection in years with lower mean temperatures. It may also be that *Ggt* infection success rate is reduced under lower temperatures although our observations suggest otherwise considering that, although infection was slower at 8°C, it did occur.

Significant correlations between rainfall and spring take-all prevalence were established However results were too variable to draw conclusions. Lucas *et al* (1997) conclude rain did not appear to be a limiting factor unless over 500mm fell in the later period. This evidence suggests that rain may only have an effect on take-all only in extreme conditions. Workings by Roget and Rovira (1991) also observed rainfall as a take-all influencing factor although this was rainfall from the previous season which would not have been picked up in our study and maybe specific to Australian climate.

As previously reported by Thomas *et al* (2010), evidence suggests that, even with a modest $1^{o}$C increase in temperature, take-all disease of wheat in the UK will become more of a concern over the next century. This chapter explores where climatic factors are important and where wheat is most at risk, for example marginal $2^{nd}/3^{rd}$ wheat in some areas could no longer be viable. Considering that there are limited management strategies for this disease, it is worth investing in research for novel ways to deal with take-all before it arises, and developing modified rotation. Climate change is a gradual process, and farming practices will adapt continually. Although this research cannot make predictions about the occurrence of take-all in specific locations, the work could form the basis for evaluating the economic impact of the disease in the future.

# Chapter 7: General discussion

Climate change is usually regarded as a future problem but weather extremes have always taken place, and are continuing to do so with perhaps more frequency (Eden, 2011; Mayes, 2006; Prior and Kendon 2011). The results presented in the previous chapters have uncovered new information that could help prepare wheat for the unknown disease challenges that the crop will be faced with over the next few decades. The results show that there are vulnerable defence periods for wheat, so climate change may increase the risk of a specific pathogen. The results could help identify a focus for wheat improvement to cope with temperature changes in current and future climates. Important insights into the temperature-sensitive nature of wheat gene *Yr36* were uncovered, along with the discovery of a previously-uncharacterised background temperature sensitivity of resistance to yellow rust that may be cultivar-dependent. Further investigation of general defence temperature sensitivity and how varying temperatures affect defence responses was explored. Influential climatic periods for take-all development were also identified.

The work on yellow rust resistance and take-all both identify vulnerable periods for wheat caused by the environment, be it weakening of host defence responses or increased threat from disease pressure. Further characterisation and understanding of vulnerable periods will be essential to control disease outbreaks under an increasingly unstable climate.

## 7.1 Increased understanding of temperature sensitivity of wheat defence

Work presented in chapter 3 showed that resistance conferred by *Yr36* is not reliant on high temperatures as previously thought (Fu *et al*., 2009; Uauy *et al*., 2005). This is consistent with observations in Segovia *et al* (*in press*), which show that *Yr36* may function in European climates. Since resistance was lost when plants experienced a drop in temperature, *Yr36* appears to be sensitive to temperature change. That said, UC1041 -*Yr36* plants experienced a similar reduction of defence responses when exposed to the same temperature decrease. It is plausible therefore, that the temperature drop affects an underlying mechanism of general resistance, rather than that specifically conferred by *Yr36*. It will be essential to see whether this observation is seen when the cultivars are

challenged with alternative isolates of *Pst* and importantly, the isolate used in the original study by Uauy *et al* (2005).

Preliminary findings suggest that *Yr39* may be similarly affected by temperature decreases, although it was not possible to confirm whether this was also seen in the Alpowa background. Nevertheless, our understanding of HTAP and resistance gene temperature sensitivity is clearly not as advanced as previously thought. Further investigation is required to determine whether HTAPs have generally been misclassified as requiring high temperature to function, or are affected by temperature changes as discovered in this study. Only one gene was found to be commonly regulated by all four resistance genotypes when transcripts were compared using microarray technology (Chen, 2013; Coram *et al*., 2008a), which suggests that defence mechanisms are not similar, perhaps due to background defence responses being responsible for HTAP-like phenotypes. Chen (2013) suggests that for breeding, clearer data on HTAP resistance can be obtained when germplasm is screened for both seedling and adult plants. However it may be more worthwhile to screen germplasm using a method similar to the ones used in chapter 3, where plants are grown in pre- and post-inoculation temperature before inoculation takes place. However, large scale screening of germplasm in this way would be difficult due to timing complications, caused by different cultivars taking different amounts of time to reach the right growth stage under different temperature regimes.

Zhu *et al* (2010) hypothesise that temperature-sensitivity in plants is largely regulated by NB-LRR coding genes rather than other signalling components. In contrast, Wang *et al* (2009) suggest that temperature resistance sensitivity to biotrophic and hemibiotrophic pathogens might be controlled by a more general mechanism. Work presented here suggests it is more in keeping with the latter theory, considering the cultivar Claire seems better adapted to coping with pathogen challenge under varying temperatures. However this observation was general to both necrotrophic and biotrophic pathogens. This is not in keeping with the hypothesis by Wang *et al* (2009), that temperature modulation of defence pathways might reflect a balance of defence against pathogens with different virulence at different temperatures. Considering only one necrotrophic pathogen was tested on Claire and Shamrock at one time, this concept still needs to be explored.

This work clearly shows that variations in temperature are affecting resistance responses, in addition to sustained average temperatures affecting resistance. In chapter 3 it was

proposed that ambient temperature increases could be priming plants for defence against *Pst.* It will be interesting to explore this idea further to define the level of temperature change and timescale necessary to induce these changes. In addition it would be interesting to know whether the increased resistance under varying temperatures seen in Claire was pre-determined by physical adaptation or whether inducible resistance mechanisms were enhanced. Precisely when temperature is affecting defence is still a mystery. Examination of meteorological records taken during field trials could help identify temperature conditions that affect resistance gene performance, and could be useful information in breeding.

Chapter 4 explores whether general temperature resilience may enable plants to cope with pathogens more effectively. It is worth investigating whether increased resilience to abiotic stresses generally makes plants more resilient to biotic stresses, as this raises the possibility that improvement of the two could be simultaneously developed. There are some studies investigating resilience in wheat to abiotic stresses (Ahmed *et al*., 2012; Mukhtar *et al*., 2010). Once identified, cultivars with good resilience to abiotic stresses could be screened for resilience to biotic stresses. As explored throughout this thesis, sensing of biotic and abiotic stresses in plants often induce common plant signalling pathways. Stress-induced signalling cascades can be in the form of changes in calcium levels, production of reactive oxygen species (ROS; Wojtaszek *et al*., 1997) and accumulation of hormones to name a few. WRKY transcription factors have been shown to play key roles in both response to biotic (Dong *et al*., 2003) and abiotic stress (Fowler and Thomashow, 2002; Mare *et al* 2004; Wang *et al*., 2013a). They are generally thought to be induced by temperatures indicative of cold hardening but their response to ambient temperature changes could be explored. Plant hormone ABA can suppress WRKY gene expression (Talanova *et al*., 2009) and, as discussed in chapters 3, 4 and 5, ABA is involved in response to both biotic and abiotic stress and a well known negative regulator of defence. ABA-mediated abiotic stress signalling takes priority over biotic stress signalling (Anderson *et al*., 2004) and considering ABA is induced by cold temperatures that are not generally associated with stress conditions (Koga *et al*., 2004a), perhaps this could be a starting point to further investigate a mechanism for how changes in temperature and thermoperiods affect defence in wheat crops.

Insight into resistance genes both involved in defence, and affected by a temperature drop in chapter 5 were not successful. What is needed is a study that combines ambient temperature changes with pathogen challenge in different backgrounds that show differences in temperature sensitivity like those observed in Shamrock and Solstice. For example both cultivars could be exposed to a temperature increase with and without pathogen challenge and transcriptomes could be analysed to look for key transcripts involved, however time points used in this investigation would need to be determined.

There are many factors that influence the effect of temperature on plant resistance. For example, the effect of a temperature change on resistance to *Pst* observed in chapter 3 may be an entirely different aspect of defence that is affected to observations in chapter 4. This was apparent from Shamrock showing defence sensitivity to temperature changes using the method in chapter 3, but not showing defence temperature sensitivity using the method described in chapter 4 (although it is worth pointing out that Shamrock did show temperature sensitivity when using the *Pst* 08/21 isolate). Also, different aspects of temperature could be affecting the same or a different aspect of defence. It could mean that throughout its life, a plant's defence will experience sensitivity to temperature changes, sensitivity to average temperature (i.e. some genes working in a particular temperature range) and in addition sensitivity to thermoperiods. On top of this, a plant will have already adapted physical barriers defences in a location before it deals with the above (Figure 7.1). With this in mind, different aspects of temperature influencing defence may be detrimental to some pathogens but beneficial to others which will add another level of complexity to an already convoluted system. Genotype x environment (G x E) interaction is recognised in the field, when results of several years are examined. Results here provide a new insight into temperature effects that could account for some of those G x E results.

## 7.2 Mapping of traits seen in the chapters 3 and 4

Whilst there are many factors that influence defence, it may still be possible to genetically map the loci that control the traits. The next logical step to advance this work would be to map traits observed in chapters 3 and 4. For observations in chapter 3, a mapping population could be developed between Soltice and Shamrock, to identify QTLs involved in the *Pst* defence temperature sensitivity observed in Shamrock. However, screening

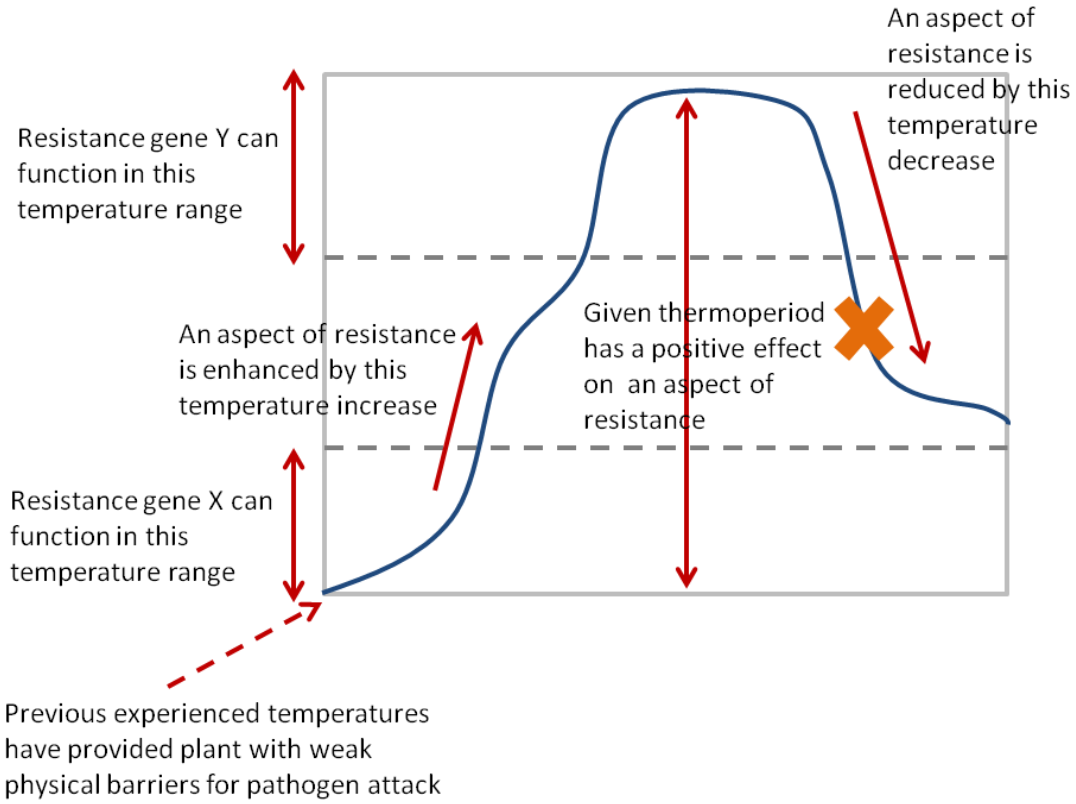



**Figure 7.1 How a plant's defence might be influenced by temperature.** Diagram to illustrate how a plant's defence mechanisms might be affected by different aspects of temperature prior to and throughout a 24hr period. The x-axis represents a 24hr time period, whilst the y-axis represents temperature. Blue line represents temperature profile throughout a 24hr period, red arrows represent temperature effects and orange cross identifies a hypothetically vulnerable defence point in the day.

seedlings developed from the crossing program would not be easy, considering the phenotype is sometimes not seen in Shamrock seedlings. However, provided parental controls were included with the offspring, it may be possible to determine that the temperature was affecting resistance in Shamrock. Another mapping population could also be developed between Claire and Shamrock, based on observations in chapter 4. However, since results were more subtle and less consistent, further experiments would first be recommended before mapping is begun, to determine that differences in temperature are responsible for the results observed in chapter 4.

## 7.3 UKCP09 to help with forecasting for both plant defence and disease forecasting

UKCP09 could provide a useful tool for making predictions about future pathogen prevalence and disease spread in the UK, considering it can provide local climate readouts. Thus, general trends about the influence of climate change on plant diseases could be made, which may affect management practices. However, this work illustrates that UKCP09 will not be appropriate for modelling how temperature affects disease responses, considering they are highly complex in nature and influenced by many factors. Limitations of UKCP09 include pathogens that are highly influenced by factors that are not included in the model. Soil pathogens like *Ggt* and *Oculimacula yallundae* (the casual agent of wheat eyespot) will be affected by soil type as well other factors. It was also a concern that UKCP09 temperature readouts being limited to air temperature would prevent below ground investigation. Results from this work, however, show that air temperature is highly correlated with soil temperature, so this should not be an issue. Models to predict take-all levels in winter wheat have been advanced (Ennaifar *et al*., 2007; Gosmel *et al*., 2013). This study indicates that these could be combined with UKCP09 climate projection models to predict outcomes, provided they accurately account for soil type. Similar models for other current and potential pathogen threats could also be combined with the most up to date UKCP interface, like those already produced by Evans *et al* (2008) and Madgwick *et al* (2011). UKCP09 visual representations act as a powerful tool for raising awareness at a local scale which will help to inform policy making decisions.

## 7.4 Overall conclusion

The most realistic approach to prepare wheat for future disease threat is through manipulation of host defence. The work in this investigation illustrates that temperature sensitivity in defence is complex, and there are many factors that can influence it. However, the work also suggests that there may be genetic variation that could enable development of more temperature-stable resistance. Whilst controlled environment experiments could help identify factors affecting defence, the results ultimately need to be applied in the field. The results indicate that greater use of environmental data from field experiments could help identify varieties from breeding programmes with defence that is more resilient to temperature changes, which will have both immediate and long term benefits for agriculture and food security. Provided accurate biological data is available, UKCP09 could also have potential in predicting long-term trends that affect diseases including those caused by soil pathogens. Preparation of wheat cultivars through breeding is a slow process (Shimelis and Laing, 2012), and we cannot prepare host defences if we do not have an idea of which pathogens will be prevalent in the future. Therefore a combination of both preparing host defence and disease forecasting of potential threats is required.

# Appendix

## A1 ANOVA tables

Table A1

| *Regression Statistics* | |
|---|---|
| Multiple R | 0.9573875 |
| R Square | 0.9165909 |
| Adjusted R Square | 0.9165835 |
| Standard Error | 1.598998 |
| Observations | 11320 |

ANOVA

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 1 | 318000.38 | 318000.38 | 124374.64 | 0.0000 |
| Residual | 11318 | 28937.8 | 2.5567945 | | |
| Total | 11319 | 346938.18 | | | |

| | *Coefficients* | *Standard Error* | *t Stat* | *P-value* | *Lower 95%* | *Upper 95%* | *Lower 95.0%* | *Upper 95.0%* |
|---|---|---|---|---|---|---|---|---|
| Intercept | 0.9351233 | 0.0293118 | 31.902582 | 6.14E-214 | 0.877667 | 0.9925796 | 0.877667 | 0.9925796 |
| X Variable 1 | 0.9208235 | 0.002611 | 352.66788 | 0 | 0.9157055 | 0.9259416 | 0.9157055 | 0.9259416 |

Table A2

| *Regression Statistics* | |
|---|---|
| Multiple R | 0.9467276 |
| R Square | 0.8962932 |
| Adjusted R Square | 0.896284 |
| Standard Error | 1.7829735 |
| Observations | 11320 |

ANOVA

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 1 | 310958.32 | 310958.32 | 97816.563 | 0.0000 |
| Residual | 11318 | 35979.861 | 3.1789946 | | |
| Total | 11319 | 346938.18 | | | |

| | *Coefficients* | *Standard Error* | *t Stat* | *P-value* | *Lower 95%* | *Upper 95%* | *Lower 95.0%* | *Upper 95.0%* |
|---|---|---|---|---|---|---|---|---|
| Intercept | -0.0670639 | 0.0357524 | -1.8757898 | 0.0607099 | -0.1371447 | 0.0030169 | -0.1371447 | 0.0030169 |
| X Variable 1 | 0.983719 | 0.0031453 | 312.7564 | 0 | 0.9775536 | 0.9898844 | 0.9775536 | 0.9898844 |

Table A3

| *Regression Statistics* | |
|---|---|
| Multiple R | 0.6661111 |
| R Square | 0.443704 |
| Adjusted R Square | 0.3818933 |
| Standard Error | 16.650221 |
| Observations | 11 |

ANOVA

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 1 | 1990.07687 | 1990.0769 | 7.17844 | 0.02523663 |
| Residual | 9 | 2495.06859 | 277.22984 | | |
| Total | 10 | 4485.14545 | | | |

| | *Coefficients* | *Standard Error* | *t Stat* | *P-value* | *Lower 95%* | *Upper 95%* | *Lower 95.0%* | *Upper 95.0%* |
|---|---|---|---|---|---|---|---|---|
| Intercept | -6.48313 | 21.72063 | -0.298478 | 0.77211 | -55.618601 | 42.6523 | -55.6186 | 42.6523 |
| X Variable 1 | 5.9562825 | 2.22311 | 2.6792605 | 0.02524 | 0.92726526 | 10.9853 | 0.927265 | 10.9853 |

Table A4

| *Regression Statistics* | |
|---|---|
| Multiple R | 0.952491 |
| R Square | 0.9072391 |
| Adjusted R Square | 0.8840489 |
| Standard Error | 12.362009 |
| Observations | 6 |

ANOVA

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 1 | 5978.5363 | 5978.5363 | 39.121614 | 0.00333204 |
| Residual | 4 | 611.27706 | 152.81926 | | |
| Total | 5 | 6589.8133 | | | |

| | *Coefficients* | *Standard Error* | *t Stat* | *P-value* | *Lower 95%* | *Upper 95%* | *Lower 95.0%* | *Upper 95.0%* |
|---|---|---|---|---|---|---|---|---|
| Intercept | -114.85623 | 27.610944 | -4.1598083 | 0.0141474 | -191.5165 | -38.1960 | -191.5165 | -38.1960 |
| X Variable 1 | 17.293896 | 2.764932 | 6.2547273 | 0.003332 | 9.6172 | 24.9706 | 9.6172 | 24.9706 |

Table A5

| *Regression Statistics* | |
|---|---|
| Multiple R | 0.8575971 |
| R Square | 0.7354728 |
| Adjusted R Square | 0.6693411 |
| Standard Error | 20.875731 |
| Observations | 6 |

ANOVA

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 1 | 4846.6288 | 4846.6288 | 11.121321 | 0.028974 |
| Residual | 4 | 1743.1845 | 435.79614 | | |
| Total | 5 | 6589.8133 | | | |

| | *Coefficients* | *Standard Error* | *t Stat* | *P-value* | *Lower 95%* | *Upper 95%* | *Lower 95.0%* | *Upper 95.0%* |
|---|---|---|---|---|---|---|---|---|
| Intercept | -20.902477 | 24.284848 | -0.860721 | 0.4379261 | -88.328023 | 46.523069 | -88.328023 | 46.523069 |
| X Variable 1 | 11.555409 | 3.465031 | 3.3348645 | 0.028974 | 1.9349405 | 21.175877 | 1.9349405 | 21.175877 |

Table A6

| *Regression Statistics* | |
|---|---|
| Multiple R | 0.853233 |
| R Square | 0.7280066 |
| Adjusted R Square | 0.6600083 |
| Standard Error | 21.116861 |
| Observations | 6 |

ANOVA

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 1 | 4774.1461 | 4774.1461 | 10.70624 | 0.0307301 |
| Residual | 4 | 1783.6873 | 445.92182 | | |
| Total | 5 | 6557.8333 | | | |

| | *Coefficients* | *Standard Error* | *t Stat* | *P-value* | *Lower 95%* | *Upper 95%* | *Lower 95.0%* | *Upper 95.0%* |
|---|---|---|---|---|---|---|---|---|
| Intercept | 123.3904 | 16.688953 | 7.3935376 | 0.0017846 | 77.054438 | 169.72636 | 77.054438 | 169.72636 |
| X Variable 1 | -0.8511602 | 0.2601314 | -3.2720391 | 0.0307301 | -1.5734008 | -0.1289196 | -1.5734008 | -0.1289196 |

Table A7

| *Regression Statistics* | |
|---|---|
| Multiple R | 0.8919862 |
| R Square | 0.7956395 |
| Adjusted R Square | 0.7275193 |
| Standard Error | 3.0090753 |
| Observations | 5 |

ANOVA

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 1 | 105.7564 | 105.7564 | 11.679938 | 0.0419168 |
| Residual | 3 | 27.163603 | 9.0545343 | | |
| Total | 4 | 132.92 | | | |

| | *Coefficients* | *Standard Error* | *t Stat* | *P-value* | *Lower 95%* | *Upper 95%* | *Lower 95.0%* | *Upper 95.0%* |
|---|---|---|---|---|---|---|---|---|
| Intercept | -13.639605 | 6.5161863 | -2.0931883 | 0.1273891 | -34.377018 | 7.0978084 | -34.377018 | 7.0978084 |
| X Variable 1 | 2.092897 | 0.6123893 | 3.4175924 | 0.0419168 | 0.144001 | 4.0417931 | 0.144001 | 4.0417931 |

Table A8

| *Regression Statistics* | |
|---|---|
| Multiple R | 0.985651 |
| R Square | 0.9715079 |
| Adjusted R Square | 0.9572619 |
| Standard Error | 1.2575581 |
| Observations | 4 |

ANOVA

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 1 | 107.8471 | 107.8471 | 68.194972 | 0.014349 |
| Residual | 2 | 3.1629046 | 1.5814523 | | |
| Total | 3 | 111.01 | | | |

| | *Coefficients* | *Standard Error* | *t Stat* | *P-value* | *Lower 95%* | *Upper 95%* | *Lower 95.0%* | *Upper 95.0%* |
|---|---|---|---|---|---|---|---|---|
| Intercept | -19.179345 | 3.3210579 | -5.7750708 | 0.0286992 | -33.468704 | -4.8899858 | -33.468704 | -4.8899858 |
| X Variable 1 | 2.4127335 | 0.2921684 | 8.2580247 | 0.014349 | 1.1556345 | 3.6698324 | 1.1556345 | 3.6698324 |

Table A9

| *Regression Statistics* | |
|---|---|
| Multiple R | 0.8733654 |
| R Square | 0.762767 |
| Adjusted R Square | 0.6836894 |
| Standard Error | 2.6352608 |
| Observations | 5 |

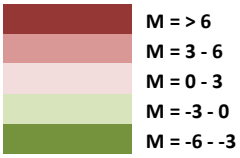
ANOVA

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 1 | 66.986202 | 66.986202 | 9.6457979 | 0.0530562 |
| Residual | 3 | 20.833798 | 6.9445994 | | |
| Total | 4 | 87.82 | | | |

| | *Coefficients* | *Standard Error* | *t Stat* | *P-value* | *Lower 95%* | *Upper 95%* | *Lower 95.0%* | *Upper 95.0%* |
|---|---|---|---|---|---|---|---|---|
| Intercept | -1.0897017 | 2.7421173 | -0.3973943 | 0.7176627 | -9.8163426 | 7.6369393 | -9.8163426 | 7.6369393 |
| X Variable 1 | 2.5570019 | 0.8233073 | 3.1057685 | 0.0530562 | -0.0631294 | 5.1771332 | -0.0631294 | 5.1771332 |

## A2 Gene expression

The table contains genes identified in chapter 5 from Laudencia-Chingcuanco *et al* (2011) with differential expression (>4 fold) in all wheat cultivars, between cold treatment of 6°C for 48 hrs and their respective control of 0 hrs. Calculation used linear modelling and an Empirical Bayes moderated *t* statistic (Smyth, 2004). M = Fold change $\log^2$, *P* = FDR adjusted *P* value. The colour represent fold change differences indicated in the diagram below



| | Spring Manitou | | Spring Northstar | | Winter Manitou | | Winter Northstar | |
|---|---|---|---|---|---|---|---|---|
| PLEXdb ID | M | *P* | M | *P* | M | *P* | M | *P* |
| Ta.1165.1.A1_at | 7.585383343 | 1.19E-22 | 7.617377778 | 7.43E-23 | 7.790669048 | 1.75E-23 | 7.210972477 | 4.11E-21 |
| Ta.2709.1.S1_s_at | 7.00760234 | 7.25E-69 | 5.445694707 | 7.62E-57 | 6.297751334 | 8.37E-64 | 3.770438226 | 4.29E-40 |
| Ta.27389.2.S1_x_at | 6.665544961 | 9.78E-71 | 5.080120228 | 6.79E-58 | 6.09682014 | 3.19E-66 | 3.519878861 | 4.48E-41 |
| Ta.18720.1.S1_x_at | 6.349273307 | 8.53E-63 | 7.193500909 | 9.44E-69 | 6.805386666 | 5.90E-66 | 5.030928622 | 3.14E-51 |
| Ta.18720.1.S1_a_at | 5.8312616 | 5.58E-64 | 6.570219894 | 1.66E-69 | 5.966459637 | 4.01E-65 | 4.922475788 | 3.38E-55 |
| Ta.245.1.S1_at | 5.694520757 | 5.72E-12 | 6.754866167 | 1.86E-15 | 5.835844868 | 1.68E-12 | 6.942826955 | 4.46E-16 |
| Ta.18720.3.S1_x_at | 5.514609426 | 2.63E-55 | 5.931410257 | 9.75E-59 | 5.459153422 | 8.01E-55 | 4.58207786 | 1.55E-46 |
| Ta.30336.1.S1_x_at | 5.189400799 | 1.90E-21 | 6.414940635 | 2.09E-28 | 5.392686054 | 1.18E-22 | 5.589205585 | 1.07E-23 |
| Ta.1138.1.S1_at | 4.718456238 | 2.89E-19 | 5.429495413 | 2.29E-23 | 4.924074661 | 1.74E-20 | 5.45426142 | 1.67E-23 |
| Ta.21768.1.S1_at | 4.693918393 | 2.31E-27 | 5.132436971 | 1.05E-30 | 4.992464273 | 1.02E-29 | 4.506009733 | 4.79E-26 |
| Ta.7934.1.S1_x_at | 4.493253363 | 3.02E-18 | 3.693076404 | 1.38E-13 | 4.651103705 | 3.18E-19 | 3.175761977 | 1.17E-10 |
| Ta.21768.1.S1_x_at | 4.492966867 | 5.20E-22 | 4.933270198 | 5.34E-25 | 5.051995834 | 8.38E-26 | 4.29720931 | 1.14E-20 |
| Ta.13134.1.A1_at | 4.487684977 | 1.38E-09 | 3.784391593 | 3.68E-07 | 4.361922437 | 3.62E-09 | 4.054722665 | 5.41E-08 |
| Ta.7934.2.S1_x_at | 4.425210521 | 2.26E-16 | 5.326142668 | 2.49E-21 | 4.164708325 | 4.69E-15 | 4.158616827 | 7.20E-15 |
| Ta.18720.2.S1_x_at | 4.373920135 | 6.85E-47 | 5.915153082 | 8.15E-61 | 5.077505965 | 1.02E-53 | 4.621478433 | 5.38E-49 |
| Ta.124.1.S1_x_at | 4.332891561 | 1.52E-15 | 4.399214331 | 8.15E-16 | 4.170962723 | 9.00E-15 | 3.735342038 | 2.71E-12 |
| Ta.13784.1.S1_at | 4.284585486 | 2.23E-12 | 5.870077782 | 1.08E-19 | 4.573309908 | 9.38E-14 | 6.040280891 | 1.70E-20 |
| Ta.27719.1.S1_at | 4.002928739 | 2.07E-35 | 3.73132492 | 1.07E-32 | 4.099577884 | 2.37E-36 | 3.364993227 | 7.36E-29 |
| Ta.27389.1.S1_at | 3.997032391 | 3.87E-25 | 5.099563835 | 7.04E-34 | 4.461841678 | 4.87E-29 | 4.557014602 | 1.30E-29 |
| Ta.7053.1.S1_at | 3.984934856 | 5.97E-10 | 3.783693555 | 4.60E-09 | 4.108983902 | 1.77E-10 | 3.873322207 | 2.39E-09 |
| Ta.613.1.S1_at | 3.935186259 | 1.46E-15 | 4.467013812 | 1.04E-18 | 4.08279246 | 1.80E-16 | 4.685405974 | 5.63E-20 |
| Ta.19327.1.S1_at | 3.903023069 | 1.77E-25 | 3.129717796 | 6.56E-19 | 3.652332058 | 2.09E-23 | 2.832801058 | 2.82E-16 |
| Ta.1722.1.S1_at | 3.882659189 | 1.09E-50 | 2.637244506 | 4.29E-34 | 3.776262269 | 1.83E-49 | 2.270606315 | 1.39E-28 |
| Ta.7934.3.S1_at | 3.842387987 | 5.71E-33 | 3.907150876 | 1.33E-33 | 3.821534066 | 7.02E-33 | 3.723580117 | 1.03E-31 |
| Ta.25026.1.S1_at | 3.813949211 | 7.57E-12 | 4.242217984 | 6.95E-14 | 4.076533289 | 3.31E-13 | 3.709455786 | 3.21E-11 |
| Ta.123.1.S1_x_at | 3.805062024 | 2.02E-18 | 5.456909628 | 7.78E-30 | 4.121885686 | 1.19E-20 | 5.636921941 | 7.67E-31 |
| TaAffx.59596.1.S1_at | 3.797261813 | 1.96E-05 | 2.351054625 | 0.01822 | 3.237548153 | 0.000331 | 2.216971613 | 0.03012 |
| Ta.21766.1.S1_at | 3.78887632 | 1.53E-14 | 3.75575127 | 2.75E-14 | 3.61914795 | 1.23E-13 | 3.389728124 | 3.89E-12 |
| Ta.22766.1.S1_a_at | 3.787589417 | 2.87E-20 | 3.67674449 | 2.02E-19 | 3.839260101 | 1.06E-20 | 3.675879433 | 2.21E-19 |
| Ta.26108.1.A1_s_at | 3.737656776 | 5.46E-05 | 3.076207948 | 0.00144 | 3.645819999 | 8.07E-05 | 3.121648837 | 0.00124 |
| Ta.8085.1.S1_at | 3.658019813 | 1.42E-16 | 4.046342201 | 3.70E-19 | 3.834721433 | 8.48E-18 | 3.821973973 | 1.39E-17 |
| Ta.13193.1.S1_at | 3.640667338 | 8.11E-35 | 3.389047958 | 4.38E-32 | 3.673622763 | 3.06E-35 | 3.107085448 | 7.72E-29 |
| Ta.14417.1.S1_at | 3.606790839 | 4.08E-09 | 2.932576463 | 2.12E-06 | 4.066480282 | 4.33E-11 | 3.368012573 | 4.99E-08 |
| Ta.28537.1.S1_x_at | 3.509728358 | 3.12E-31 | 4.948534768 | 2.45E-45 | 3.77155557 | 4.18E-34 | 4.757540497 | 1.88E-43 |

| | Spring Manitou | | Spring Northstar | | Winter Manitou | | Winter Northstar | |
|---|---|---|---|---|---|---|---|---|
| PLEXdb ID | M | *P* | M | *P* | M | *P* | M | *P* |
| Ta.145.1.A1_x_at | 3.501307629 | 8.01E-12 | 4.814402731 | 5.43E-19 | 3.861707152 | 8.14E-14 | 4.998644631 | 5.48E-20 |
| Ta.2541.1.S1_x_at | 3.368822998 | 5.91E-17 | 3.765222047 | 7.51E-20 | 3.757747862 | 6.75E-20 | 3.728504438 | 1.46E-19 |
| Ta.13239.1.S1_at | 3.278996175 | 1.55E-12 | 3.705997214 | 4.87E-15 | 3.519767059 | 4.75E-14 | 3.991697344 | 9.53E-17 |
| TaAffx.111791.1.S1_at | 3.275380171 | 4.82E-22 | 2.376380864 | 7.96E-14 | 3.075148308 | 2.76E-20 | 2.453434107 | 1.64E-14 |
| Ta.10933.1.S1_at | 3.215952556 | 6.66E-09 | 3.065645253 | 3.77E-08 | 3.300080955 | 2.60E-09 | 3.002575233 | 7.78E-08 |
| Ta.30798.3.S1_at | 3.153974466 | 5.78E-17 | 3.141771739 | 8.40E-17 | 3.369222271 | 9.90E-19 | 3.22769617 | 1.81E-17 |
| TaAffx.73215.1.S1_at | 3.14573993 | 1.53E-35 | 2.824549103 | 1.59E-31 | 3.16131864 | 8.10E-36 | 2.267118756 | 7.85E-24 |
| Ta.865.2.A1_at | 3.120164239 | 1.79E-08 | 3.787968625 | 1.68E-11 | 3.435449179 | 6.19E-10 | 3.809036517 | 1.45E-11 |
| Ta.23419.1.S1_x_at | 3.106451691 | 1.17E-06 | 3.624118547 | 1.55E-08 | 3.428091711 | 7.06E-08 | 3.092057911 | 1.66E-06 |
| Ta.10408.1.S1_at | 3.09181041 | 3.19E-12 | 2.425243204 | 3.32E-08 | 2.553085879 | 4.94E-09 | 2.094403744 | 2.20E-06 |
| Ta.2541.1.S1_s_at | 3.055342902 | 3.32E-25 | 3.136104918 | 3.48E-26 | 3.27711911 | 1.10E-27 | 2.893588395 | 1.66E-23 |
| Ta.28273.1.S1_x_at | 3.020347491 | 4.68E-08 | 2.618830994 | 2.74E-06 | 2.911624549 | 1.32E-07 | 2.271860003 | 6.60E-05 |
| Ta.13183.1.S1_x_at | 3.012507982 | 1.47E-07 | 4.78936453 | 1.20E-15 | 3.364916306 | 4.26E-09 | 5.42469812 | 8.66E-19 |
| Ta.9600.1.S1_x_at | 2.969572432 | 0.002247 | 2.795087891 | 0.005053 | 2.889015809 | 0.002908 | 2.428161781 | 0.02052 |
| TaAffx.19033.1.S1_at | 2.933082778 | 2.99E-05 | 2.586236158 | 0.000324 | 3.089520349 | 8.96E-06 | 2.680991056 | 0.00019 |
| Ta.345.1.S1_x_at | 2.925612579 | 2.46E-13 | 2.909196501 | 3.58E-13 | 3.010539278 | 5.20E-14 | 3.224841033 | 2.24E-15 |
| Ta.18574.1.A1_x_at | 2.919847568 | 1.87E-06 | 4.161336597 | 1.86E-11 | 3.300145168 | 6.01E-08 | 4.327934789 | 3.89E-12 |
| Ta.6792.1.S1_at | 2.893354812 | 3.75E-14 | 2.30490339 | 8.01E-10 | 2.961644989 | 9.78E-15 | 2.044891438 | 4.92E-08 |
| Ta.7091.1.S1_at | 2.879651283 | 1.24E-07 | 2.463051881 | 8.10E-06 | 2.933360446 | 6.76E-08 | 2.436367926 | 1.14E-05 |
| Ta.345.1.S1_at | 2.878758996 | 1.10E-11 | 3.11631112 | 3.74E-13 | 3.116293006 | 2.83E-13 | 3.317358222 | 1.91E-14 |
| Ta.3140.1.S1_at | 2.822684211 | 2.87E-14 | 2.166040964 | 2.43E-09 | 2.656311604 | 4.41E-13 | 2.092197654 | 9.17E-09 |
| TaAffx.129374.2.S1_x_at | 2.81920948 | 1.23E-06 | 3.488521568 | 2.28E-09 | 2.905331346 | 5.22E-07 | 3.873740231 | 4.65E-11 |
| TaAffx.131747.1.S1_x_at | 2.815267065 | 0.00027 | 3.977368171 | 1.41E-07 | 2.723944141 | 0.000424 | 4.318028956 | 1.25E-08 |
| Ta.30338.1.S1_x_at | 2.809771029 | 6.29E-31 | 2.731200881 | 5.30E-30 | 2.904783383 | 2.53E-32 | 2.361007214 | 7.49E-25 |
| Ta.27725.1.S1_at | 2.800076553 | 9.83E-07 | 2.488831219 | 1.79E-05 | 2.88130032 | 4.34E-07 | 2.217326996 | 0.000181 |
| Ta.19303.1.S1_at | 2.739701617 | 3.54E-08 | 3.998200015 | 8.80E-15 | 2.742073671 | 3.35E-08 | 3.78331293 | 1.41E-13 |
| Ta.8037.1.A1_at | 2.711532595 | 0.00088 | 4.082388574 | 2.08E-07 | 3.20888715 | 4.91E-05 | 4.393680843 | 2.49E-08 |
| TaAffx.56641.1.A1_at | 2.672808751 | 3.39E-06 | 2.523116584 | 1.42E-05 | 2.146883057 | 0.000248 | 2.06242418 | 0.000600 |
| Ta.22764.1.S1_x_at | 2.650736212 | 1.27E-10 | 2.453333977 | 2.76E-09 | 2.790737447 | 1.49E-11 | 2.318377737 | 2.08E-08 |
| Ta.13595.1.A1_at | 2.635095772 | 7.36E-22 | 3.084958634 | 5.33E-27 | 2.760437391 | 2.77E-23 | 2.890114555 | 9.82E-25 |
| Ta.13183.1.S1_s_at | 2.621782242 | 4.69E-07 | 4.157455388 | 1.17E-14 | 3.013540363 | 6.62E-09 | 4.551894242 | 9.72E-17 |
| Ta.22063.1.S1_s_at | 2.612968725 | 1.71E-05 | 2.724266893 | 7.69E-06 | 2.529883555 | 3.02E-05 | 2.559158669 | 3.22E-05 |
| TaAffx.97142.1.S1_at | 2.604098868 | 2.04E-11 | 2.781412501 | 1.47E-12 | 2.908086925 | 1.36E-13 | 2.702614036 | 5.85E-12 |
| TaAffx.34169.1.S1_at | 2.578404579 | 1.69E-05 | 2.133691347 | 0.000567 | 2.925746578 | 7.82E-07 | 2.560185544 | 2.39E-05 |

| | Spring Manitou | | Spring Northstar | | Winter Manitou | | Winter Northstar | |
|---|---|---|---|---|---|---|---|---|
| PLEXdb ID | M | *P* | M | *P* | M | *P* | M | *P* |
| Ta.28369.1.S1_at | 2.514804932 | 2.83E-05 | 2.76015723 | 4.15E-06 | 2.664307646 | 7.49E-06 | 2.558910842 | 2.44E-05 |
| Ta.6380.1.A1_at | 2.487081646 | 4.21E-07 | 2.821561462 | 1.11E-08 | 2.398202336 | 1.05E-06 | 2.717750053 | 4.16E-08 |
| Ta.12663.1.S1_at | 2.475880999 | 1.34E-05 | 2.560035028 | 7.20E-06 | 2.691972376 | 1.77E-06 | 2.494931079 | 1.41E-05 |
| Ta.8190.1.S1_at | 2.474829753 | 9.58E-07 | 2.656036863 | 1.60E-07 | 2.767784715 | 3.81E-08 | 2.646998999 | 1.94E-07 |
| Ta.6379.1.S1_at | 2.467470602 | 0.000117 | 2.716830442 | 2.04E-05 | 2.747273933 | 1.31E-05 | 2.605497753 | 5.28E-05 |
| Ta.5749.2.S1_at | 2.460896716 | 9.88E-12 | 2.298780344 | 1.93E-10 | 2.271270022 | 2.28E-10 | 2.255361066 | 4.39E-10 |
| TaAffx.65807.1.A1_at | 2.448825872 | 3.39E-12 | 2.468185829 | 2.80E-12 | 2.55095027 | 4.49E-13 | 2.447059107 | 4.65E-12 |
| Ta.14348.1.S1_at | 2.445456903 | 4.40E-14 | 2.118224639 | 3.44E-11 | 2.31907008 | 4.57E-13 | 2.114726361 | 4.01E-11 |
| Ta.1909.1.A1_at | 2.431169067 | 3.70E-06 | 2.614505561 | 6.91E-07 | 2.503700374 | 1.68E-06 | 2.409441401 | 5.74E-06 |
| Ta.759.1.S1_at | 2.39553581 | 1.78E-05 | 2.461762094 | 1.11E-05 | 2.540270123 | 4.39E-06 | 2.432288136 | 1.59E-05 |
| TaAffx.54307.1.S1_x_at | 2.36496178 | 2.42E-06 | 3.67786215 | 6.16E-13 | 2.89441595 | 6.66E-09 | 3.392066197 | 2.42E-11 |
| Ta.23758.1.S1_x_at | 2.353247517 | 3.92E-05 | 2.982449637 | 1.44E-07 | 2.304631163 | 5.38E-05 | 2.61499467 | 4.99E-06 |
| TaAffx.71465.1.S1_at | 2.329142942 | 3.42E-05 | 2.997125053 | 7.46E-08 | 2.744796578 | 7.14E-07 | 3.476578248 | 5.69E-10 |
| TaAffx.17284.1.A1_at | 2.211625211 | 1.40E-19 | 4.79794385 | 1.54E-48 | 2.4048556 | 4.91E-22 | 4.380262843 | 2.97E-44 |
| Ta.10148.1.S1_at | 2.204760048 | 1.57E-09 | 2.09757995 | 1.05E-08 | 2.285606918 | 4.02E-10 | 2.010895175 | 4.53E-08 |
| Ta.26928.1.S1_x_at | 2.190687209 | 2.55E-05 | 3.031868364 | 4.45E-09 | 2.308735778 | 7.49E-06 | 2.850967743 | 3.78E-08 |
| Ta.18362.1.A1_at | 2.185391411 | 3.03E-06 | 3.248180156 | 6.93E-12 | 2.138298507 | 4.62E-06 | 2.391534417 | 3.72E-07 |
| Ta.26929.2.S1_a_at | 2.172817078 | 4.84E-06 | 3.288827521 | 7.52E-12 | 2.285169098 | 1.33E-06 | 2.980559666 | 4.65E-10 |
| TaAffx.95521.1.S1_at | 2.172614381 | 0.000824 | 3.372664541 | 7.32E-08 | 2.513589337 | 6.85E-05 | 3.997187135 | 2.41E-10 |
| Ta.25860.1.A1_at | 2.118049447 | 3.55E-15 | 2.541973934 | 1.02E-19 | 2.550975929 | 6.53E-20 | 2.044923969 | 2.55E-14 |
| Ta.345.2.S1_x_at | 2.052065893 | 1.25E-11 | 2.302509448 | 8.09E-14 | 2.204240929 | 4.53E-13 | 2.495568694 | 1.40E-15 |
| Ta.28378.2.S1_at | 2.030951934 | 1.85E-15 | 3.067004018 | 2.19E-27 | 2.205876432 | 1.74E-17 | 2.816571114 | 1.87E-24 |
| TaAffx.98930.1.A1_at | 2.006149513 | 2.88E-17 | 2.810924094 | 2.03E-27 | 3.308560847 | 2.09E-33 | 2.771555752 | 7.89E-27 |
| Ta.6665.3.S1_x_at | -2.030984573 | 7.61E-11 | -2.183995772 | 4.48E-12 | -2.149850654 | 6.73E-12 | -2.18369617 | 5.26E-12 |
| Ta.9320.1.S1_at | -2.032267261 | 0.004758 | -2.674359514 | 0.000103 | -2.678655398 | 8.31E-05 | -2.255486045 | 0.001602 |
| Ta.9216.2.A1_at | -2.052869879 | 9.69E-21 | -3.073330692 | 1.84E-34 | -2.842101529 | 1.08E-31 | -3.502294333 | 8.20E-40 |
| Ta.27217.1.S1_at | -2.056254424 | 9.20E-05 | -2.062483648 | 9.72E-05 | -2.126307354 | 4.50E-05 | -2.059016181 | 0.000111 |
| Ta.9216.2.A1_x_at | -2.056860344 | 1.23E-24 | -2.3031322 | 1.41E-28 | -2.140809611 | 4.85E-26 | -2.614801561 | 4.43E-33 |
| Ta.3455.1.S1_at | -2.062477321 | 1.96E-07 | -2.33694139 | 4.56E-09 | -2.148374866 | 5.63E-08 | -2.019362617 | 4.57E-07 |
| Ta.12226.1.S1_x_at | -2.068911219 | 6.34E-06 | -2.028207297 | 1.12E-05 | -2.273326839 | 5.75E-07 | -2.035397445 | 1.15E-05 |
| Ta.1677.1.S1_at | -2.123019431 | 2.99E-06 | -2.83235327 | 5.53E-10 | -2.349125249 | 1.99E-07 | -2.136645926 | 3.19E-06 |
| Ta.19357.1.S1_at | -2.131514596 | 6.07E-20 | -2.260378078 | 1.24E-21 | -2.401537551 | 1.92E-23 | -2.06424449 | 5.36E-19 |
| Ta.6971.2.S1_a_at | -2.147922999 | 2.84E-08 | -2.215740855 | 1.23E-08 | -2.358552229 | 1.14E-09 | -2.183870782 | 2.21E-08 |
| Ta.21174.1.S1_at | -2.165767562 | 6.70E-08 | -2.299533743 | 1.18E-08 | -2.442425561 | 1.18E-09 | -2.059791262 | 3.69E-07 |

| | Spring Manitou | | Spring Northstar | | Winter Manitou | | Winter Northstar | |
|---|---|---|---|---|---|---|---|---|
| PLEXdb ID | M | *P* | M | *P* | M | *P* | M | *P* |
| Ta.9216.1.A1_x_at | -2.167539378 | 1.14E-11 | -2.715796938 | 2.45E-16 | -2.712325721 | 1.94E-16 | -2.590365093 | 3.05E-15 |
| Ta.28866.1.S1_at | -2.181092944 | 1.84E-25 | -2.592659632 | 1.14E-31 | -2.666787992 | 8.20E-33 | -2.657973278 | 1.79E-32 |
| Ta.185.1.S1_at | -2.183048603 | 2.50E-26 | -2.508700691 | 2.07E-31 | -2.798398098 | 1.34E-35 | -2.376364499 | 2.77E-29 |
| Ta.15228.1.S1_at | -2.188988478 | 1.61E-07 | -2.356718634 | 1.98E-08 | -2.541540942 | 1.22E-09 | -2.368725512 | 1.91E-08 |
| Ta.8612.1.S1_at | -2.191546117 | 4.10E-13 | -2.452708458 | 1.78E-15 | -2.568141868 | 1.14E-16 | -2.441404832 | 2.26E-15 |
| Ta.15228.1.S1_x_at | -2.195222718 | 6.22E-08 | -2.4912655 | 1.12E-09 | -2.475384458 | 1.09E-09 | -2.533008977 | 6.72E-10 |
| Ta.9216.1.A1_a_at | -2.196053529 | 2.28E-09 | -2.531040906 | 1.12E-11 | -2.288097445 | 4.93E-10 | -2.242925105 | 1.49E-09 |
| Ta.3698.2.S1_x_at | -2.200259098 | 8.20E-19 | -2.445101017 | 8.46E-22 | -2.536167003 | 6.49E-23 | -2.544446031 | 5.50E-23 |
| Ta.12704.1.S1_at | -2.20106885 | 3.14E-16 | -2.112798356 | 3.25E-15 | -2.267615113 | 5.35E-17 | -2.47183726 | 3.88E-19 |
| Ta.8661.2.S1_a_at | -2.210256314 | 9.72E-07 | -2.224174566 | 9.72E-07 | -2.280174905 | 4.01E-07 | -2.167885478 | 2.01E-06 |
| Ta.9320.1.S1_x_at | -2.222743112 | 0.002977 | -2.602519575 | 0.000372 | -2.833850603 | 7.07E-05 | -2.813137058 | 0.000109 |
| TaAffx.4230.1.A1_at | -2.253152504 | 2.09E-07 | -2.678017809 | 9.53E-10 | -2.553883241 | 3.90E-09 | -2.202179336 | 5.07E-07 |
| TaAffx.15937.1.S1_at | -2.261890114 | 1.18E-05 | -2.575719855 | 5.74E-07 | -3.295501486 | 1.39E-10 | -2.523372917 | 1.08E-06 |
| Ta.21286.1.S1_at | -2.273138837 | 4.50E-19 | -2.303935701 | 2.12E-19 | -2.334080113 | 7.60E-20 | -2.217659081 | 2.43E-18 |
| TaAffx.55684.1.S1_x_at | -2.385710747 | 1.47E-05 | -2.185497639 | 9.27E-05 | -2.514188753 | 4.11E-06 | -2.107730385 | 0.000194 |
| Ta.12686.2.S1_at | -2.389391061 | 3.28E-12 | -2.13561396 | 3.90E-10 | -2.674465666 | 1.35E-14 | -2.360010819 | 7.28E-12 |
| Ta.10774.1.A1_at | -2.419724758 | 0.004880 | -2.237945024 | 0.012215 | -2.809328703 | 0.000690 | -2.151454169 | 0.017937 |
| Ta.14699.1.S1_at | -2.420260371 | 2.20E-07 | -2.508573715 | 9.18E-08 | -2.448464765 | 1.50E-07 | -2.099001478 | 1.02E-05 |
| Ta.4041.1.S1_x_at | -2.479476574 | 1.65E-07 | -2.574844386 | 6.43E-08 | -2.366400662 | 5.73E-07 | -2.677443304 | 2.10E-08 |
| Ta.8108.2.S1_x_at | -2.48061328 | 0.002722 | -2.235970931 | 0.009523 | -2.366245509 | 0.004365 | -2.049537346 | 0.021565 |
| Ta.8108.2.S1_at | -2.504394918 | 0.00192 | -2.199456763 | 0.009288 | -2.631433066 | 0.000919 | -2.171237163 | 0.010977 |
| Ta.96.1.S1_at | -2.541225408 | 8.22E-15 | -2.55068467 | 8.26E-15 | -2.267960204 | 1.66E-12 | -2.571720408 | 5.30E-15 |
| Ta.22933.1.S1_at | -2.572911864 | 9.79E-09 | -2.7059685 | 2.13E-09 | -2.639973454 | 3.92E-09 | -2.008075779 | 1.12E-05 |
| Ta.18814.2.S1_at | -2.583788652 | 5.70E-11 | -2.356307632 | 2.31E-09 | -2.730874771 | 5.35E-12 | -2.149206902 | 5.30E-08 |
| Ta.6167.1.S1_at | -2.587461926 | 1.25E-11 | -2.43848431 | 1.71E-10 | -2.4651694 | 8.15E-11 | -2.209677468 | 6.90E-09 |
| Ta.25274.1.A1_x_at | -2.599667688 | 3.79E-11 | -2.999506072 | 8.07E-14 | -2.346963952 | 1.79E-09 | -2.745020441 | 5.33E-12 |
| TaAffx.31514.1.S1_at | -2.626882513 | 2.34E-06 | -2.440893794 | 1.43E-05 | -2.876474834 | 2.01E-07 | -2.436250916 | 1.65E-05 |
| Ta.4973.2.S1_a_at | -2.652336064 | 7.52E-11 | -2.058505622 | 4.40E-07 | -2.961368266 | 6.06E-13 | -2.16542655 | 1.14E-07 |
| Ta.30523.1.S1_at | -2.657778205 | 3.33E-29 | -2.712136508 | 4.56E-30 | -3.139865409 | 1.33E-35 | -2.478718913 | 8.52E-27 |
| Ta.21052.1.S1_x_at | -2.693874545 | 3.88E-07 | -3.511729878 | 6.39E-11 | -2.713157134 | 3.04E-07 | -3.463328381 | 1.25E-10 |
| Ta.12590.1.S1_x_at | -2.72049501 | 0.000211 | -2.896185786 | 7.81E-05 | -3.216918241 | 7.34E-06 | -2.695819858 | 0.000295 |
| Ta.24542.1.S1_at | -2.726258591 | 1.94E-19 | -2.43598492 | 2.00E-16 | -2.530689222 | 1.69E-17 | -2.085731414 | 5.43E-13 |
| TaAffx.53974.1.S1_at | -2.736801368 | 6.80E-05 | -2.603539175 | 0.000186 | -2.810338437 | 3.77E-05 | -2.77414392 | 6.60E-05 |
| Ta.23362.2.S1_at | -2.745710329 | 4.14E-07 | -2.193097645 | 7.93E-05 | -2.758748715 | 3.47E-07 | -2.134471624 | 0.00014 |

| | Spring Manitou | | Spring Northstar | | Winter Manitou | | Winter Northstar | |
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| Ta.4200.1.A1_at | -2.75697554 | 1.17E-11 | -2.145205282 | 1.10E-07 | -2.868847178 | 1.85E-12 | -2.066659404 | 3.51E-07 |
| Ta.7773.1.S1_x_at | -2.773873891 | 3.33E-07 | -2.435329498 | 9.84E-06 | -2.820263729 | 2.01E-07 | -2.057223275 | 0.000274 |
| TaAffx.55684.1.S1_at | -2.774417828 | 2.12E-06 | -2.482828508 | 2.88E-05 | -2.745803588 | 2.50E-06 | -2.41350405 | 5.62E-05 |
| Ta.23362.3.S1_x_at | -2.775749989 | 5.30E-07 | -2.506898356 | 7.63E-06 | -3.093011178 | 2.14E-08 | -2.494186667 | 9.43E-06 |
| TaAffx.722.1.S1_at | -2.784457327 | 3.34E-06 | -2.427041279 | 7.04E-05 | -2.877551823 | 1.38E-06 | -2.315199058 | 0.000181 |
| TaAffx.102561.1.S1_s_at | -2.8341063 | 7.61E-09 | -2.213680404 | 8.48E-06 | -3.101691872 | 2.86E-10 | -2.37513125 | 1.73E-06 |
| Ta.5484.1.A1_x_at | -2.841106652 | 1.54E-24 | -2.713528253 | 4.05E-23 | -2.669991598 | 1.18E-22 | -2.493461381 | 1.53E-20 |
| Ta.12686.1.A1_at | -2.846097501 | 6.16E-16 | -2.360281791 | 5.98E-12 | -2.860476128 | 4.02E-16 | -2.542320715 | 2.28E-13 |
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| Ta.30807.2.S1_s_at | -2.861573759 | 3.27E-09 | -2.784083226 | 9.90E-09 | -3.20774451 | 4.23E-11 | -2.787760106 | 1.09E-08 |
| Ta.1454.2.S1_x_at | -2.876799802 | 1.47E-08 | -2.70822416 | 1.15E-07 | -3.109545202 | 9.69E-10 | -2.39000069 | 3.49E-06 |
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| Ta.9255.1.S1_at | -2.974734191 | 9.29E-19 | -2.721205297 | 2.15E-16 | -3.060397257 | 1.45E-19 | -2.481650551 | 2.64E-14 |
| Ta.4834.1.S1_at | -3.036599989 | 0.000406 | -2.608531261 | 0.003547 | -3.067361142 | 0.000318 | -2.95589627 | 0.000724 |
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| Ta.12530.1.S1_at | -3.066488883 | 6.86E-13 | -2.338177303 | 2.71E-08 | -3.282072735 | 2.24E-14 | -2.198102028 | 1.94E-07 |
| TaAffx.105801.1.S1_s_at | -3.068020873 | 9.91E-11 | -2.754303639 | 6.38E-09 | -3.296119299 | 4.86E-12 | -2.595174512 | 4.89E-08 |
| TaAffx.28156.1.S1_at | -3.071806027 | 1.01E-08 | -3.073058774 | 1.19E-08 | -2.883589721 | 7.06E-08 | -2.514021035 | 3.84E-06 |
| Ta.10.2.S1_x_at | -3.076411691 | 2.74E-08 | -3.643053783 | 8.07E-11 | -3.753319827 | 1.77E-11 | -3.211206053 | 9.28E-09 |
| Ta.11171.1.A1_at | -3.076454731 | 2.65E-10 | -2.623515665 | 7.46E-08 | -2.864230392 | 3.49E-09 | -2.293892049 | 3.12E-06 |
| Ta.4041.1.S1_at | -3.076669243 | 9.91E-09 | -2.631239517 | 1.18E-06 | -2.929715731 | 4.62E-08 | -2.75933566 | 3.57E-07 |
| Ta.20175.1.A1_at | -3.080911362 | 1.26E-06 | -4.215250095 | 5.37E-11 | -3.586069751 | 1.48E-08 | -4.335793952 | 1.79E-11 |
| TaAffx.499.1.A1_at | -3.089784492 | 0.000244 | -3.013642113 | 0.000406 | -3.576639288 | 1.40E-05 | -2.685739426 | 0.002211 |
| Ta.24635.1.S1_at | -3.107791411 | 9.00E-07 | -2.688286538 | 2.91E-05 | -3.080164004 | 1.05E-06 | -2.028748447 | 0.002833 |
| Ta.1875.1.S1_at | -3.109743817 | 2.70E-07 | -2.750324004 | 7.02E-06 | -3.357922411 | 2.65E-08 | -2.712703874 | 1.05E-05 |
| Ta.16135.1.A1_at | -3.113101491 | 3.76E-05 | -2.228655934 | 0.006063 | -3.296152204 | 1.03E-05 | -2.026274967 | 0.016062 |
| Ta.2827.1.S1_x_at | -3.117614611 | 8.95E-36 | -2.830661338 | 3.82E-32 | -3.418345255 | 1.38E-39 | -2.51302118 | 8.76E-28 |
| Ta.11919.2.S1_x_at | -3.117906884 | 2.15E-09 | -2.690792223 | 2.71E-07 | -3.114954509 | 2.10E-09 | -2.149907096 | 5.85E-05 |
| Ta.14545.1.S1_at | -3.148982465 | 6.90E-05 | -2.125306654 | 0.015011 | -2.636700375 | 0.001117 | -1.116077752 | 0.362813 |
| Ta.9255.1.S1_a_at | -3.15687506 | 1.63E-17 | -2.78031876 | 1.76E-14 | -3.061616016 | 8.32E-17 | -2.597090468 | 5.17E-13 |
| Ta.5484.1.A1_a_at | -3.157017731 | 5.17E-35 | -3.516084541 | 2.44E-39 | -3.230974837 | 6.36E-36 | -3.402211576 | 5.46E-38 |
| Ta.23362.1.S1_x_at | -3.1713128 | 9.00E-10 | -2.400157764 | 4.29E-06 | -3.145530566 | 1.15E-09 | -2.595384546 | 6.58E-07 |
| Ta.28584.1.A1_at | -3.179991799 | 9.75E-05 | -2.962683936 | 0.000362 | -3.312237214 | 4.15E-05 | -2.92097953 | 0.000486 |

| | Spring Manitou | | Spring Northstar | | Winter Manitou | | Winter Northstar | |
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| TaAffx.2601.1.S1_at | -3.188259821 | 7.54E-08 | -2.897565845 | 1.28E-06 | -3.132503969 | 1.20E-07 | -2.675391614 | 9.42E-06 |
| TaAffx.837.1.S1_x_at | -3.214206959 | 2.83E-15 | -3.701907351 | 9.46E-19 | -3.693588053 | 9.33E-19 | -3.45774615 | 6.54E-17 |
| Ta.19142.1.S1_at | -3.22477415 | 5.24E-07 | -2.255493307 | 0.000818 | -3.24263817 | 4.27E-07 | -2.399187006 | 0.000340 |
| Ta.5118.3.S1_x_at | -3.241794257 | 7.42E-13 | -3.11452427 | 4.87E-12 | -3.304533593 | 2.56E-13 | -2.526616346 | 1.62E-08 |
| Ta.20649.2.S1_a_at | -3.255836608 | 3.15E-10 | -2.575931985 | 7.17E-07 | -3.40662468 | 5.24E-11 | -2.231473581 | 2.31E-05 |
| Ta.27596.1.S1_x_at | -3.27670296 | 1.37E-18 | -2.655086538 | 1.46E-13 | -3.51697721 | 1.41E-20 | -2.361348861 | 3.07E-11 |
| Ta.7406.1.S1_x_at | -3.282369352 | 2.14E-09 | -3.628991087 | 6.02E-11 | -3.569469656 | 8.54E-11 | -3.027405805 | 4.19E-08 |
| Ta.11919.2.S1_a_at | -3.286676817 | 2.72E-07 | -2.762367427 | 2.14E-05 | -3.362030993 | 1.36E-07 | -2.370490171 | 0.000381 |
| Ta.8733.2.S1_a_at | -3.296166928 | 2.64E-08 | -2.658177793 | 9.69E-06 | -3.351951957 | 1.48E-08 | -2.739193208 | 5.35E-06 |
| Ta.13152.3.S1_x_at | -3.300286193 | 5.35E-09 | -3.744555994 | 6.06E-11 | -3.668170372 | 1.02E-10 | -3.359989202 | 3.99E-09 |
| Ta.23032.2.S1_x_at | -3.308418277 | 3.82E-15 | -3.144424247 | 5.99E-14 | -3.461867536 | 3.11E-16 | -3.127254491 | 8.25E-14 |
| Ta.2631.3.S1_x_at | -3.320661278 | 1.72E-07 | -2.35079636 | 0.000369 | -3.199799048 | 4.61E-07 | -2.21964221 | 0.000938 |
| Ta.12252.1.S1_s_at | -3.322248153 | 1.64E-11 | -2.138245223 | 1.63E-05 | -3.392209372 | 6.13E-12 | -2.314648127 | 2.95E-06 |
| Ta.27596.1.S1_at | -3.337645948 | 7.49E-17 | -2.709384324 | 3.02E-12 | -3.559620327 | 1.48E-18 | -2.613919428 | 1.56E-11 |
| Ta.1454.2.S1_at | -3.339864282 | 1.63E-09 | -3.14173921 | 1.57E-08 | -3.621757948 | 7.20E-11 | -2.657012341 | 2.09E-06 |
| Ta.526.1.S1_x_at | -3.357982753 | 4.33E-29 | -2.292965591 | 2.24E-17 | -3.478906521 | 1.62E-30 | -2.280267494 | 3.36E-17 |
| Ta.19627.1.S1_at | -3.362060228 | 1.61E-12 | -3.09481189 | 6.44E-11 | -3.845497439 | 2.01E-15 | -3.134812173 | 4.15E-11 |
| Ta.5118.1.S1_s_at | -3.390392637 | 3.02E-11 | -3.253420854 | 2.01E-10 | -3.351553618 | 4.68E-11 | -2.77569217 | 5.33E-08 |
| Ta.8700.1.S1_at | -3.396351015 | 7.57E-12 | -3.169974106 | 1.55E-10 | -3.838189355 | 2.28E-14 | -2.938620165 | 2.92E-09 |
| Ta.13988.1.S1_at | -3.431178424 | 0.002822 | -3.169790401 | 0.007651 | -3.648730612 | 0.001199412 | -2.745786102 | 0.028596 |
| Ta.3696.2.S1_at | -3.483724316 | 1.03E-11 | -2.660868873 | 1.71E-07 | -3.455294466 | 1.31E-11 | -2.470768403 | 1.41E-06 |
| TaAffx.68872.1.S1_at | -3.484212096 | 2.59E-06 | -2.408402816 | 0.002196 | -3.610266029 | 9.85E-07 | -2.394279715 | 0.002507 |
| Ta.4916.1.S1_at | -3.536915813 | 1.86E-11 | -3.269258665 | 5.30E-10 | -3.478733894 | 3.51E-11 | -3.052711795 | 6.82E-09 |
| Ta.9492.1.S1_at | -3.568090001 | 2.34E-05 | -2.368185501 | 0.010406 | -4.038922507 | 1.26E-06 | -2.141484832 | 0.026027 |
| Ta.8733.1.S1_at | -3.596499635 | 3.51E-08 | -2.632193994 | 8.32E-05 | -3.874684614 | 2.83E-09 | -2.664454984 | 7.42E-05 |
| Ta.4593.1.A1_at | -3.653919406 | 9.46E-10 | -3.051275939 | 3.58E-07 | -3.565402446 | 2.15E-09 | -2.765980592 | 4.84E-06 |
| Ta.27799.1.A1_s_at | -3.700185676 | 6.61E-16 | -2.763118234 | 4.78E-10 | -3.491899727 | 1.12E-14 | -2.542902734 | 9.93E-09 |
| Ta.10183.1.S1_at | -3.729550914 | 3.25E-15 | -3.948373478 | 1.87E-16 | -4.065524855 | 2.65E-17 | -3.535655906 | 5.98E-14 |
| Ta.7406.2.S1_at | -3.736337847 | 2.78E-13 | -3.638031787 | 1.11E-12 | -4.014265944 | 6.95E-15 | -3.711228932 | 4.75E-13 |
| TaAffx.81146.1.S1_at | -3.744881667 | 4.59E-14 | -3.104113852 | 2.06E-10 | -3.591000108 | 2.92E-13 | -3.004550028 | 7.77E-10 |
| Ta.2631.1.S1_at | -3.74522406 | 1.09E-07 | -3.040637339 | 2.24E-05 | -3.761422889 | 9.10E-08 | -2.979622617 | 3.77E-05 |
| Ta.12896.1.S1_at | -3.749474602 | 6.99E-15 | -3.131734114 | 3.37E-11 | -3.510099963 | 1.53E-13 | -2.53768958 | 6.56E-08 |
| Ta.7406.2.S1_x_at | -3.809963419 | 9.81E-13 | -3.721070358 | 3.30E-12 | -4.107100008 | 2.36E-14 | -3.875029083 | 5.59E-13 |

| | Spring Manitou | | Spring Northstar | | Winter Manitou | | Winter Northstar | |
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| PLEXdb ID | M | *P* | M | *P* | M | *P* | M | *P* |
| Ta.24155.2.S1_x_at | -3.82285985 | 2.66E-47 | -2.181668248 | 3.01E-25 | -3.892399955 | 3.47E-48 | -2.459581311 | 2.77E-29 |
| Ta.23032.1.S1_at | -3.847460443 | 5.32E-24 | -3.314483231 | 1.47E-19 | -3.456484241 | 8.44E-21 | -2.933729688 | 2.52E-16 |
| Ta.6098.1.S1_at | -3.881081787 | 3.81E-10 | -3.90975168 | 3.80E-10 | -3.959168681 | 1.71E-10 | -3.382922506 | 5.65E-08 |
| Ta.12741.1.S1_s_at | -3.89693426 | 1.20E-08 | -3.19773517 | 3.72E-06 | -3.886326824 | 1.26E-08 | -3.062122826 | 1.11E-05 |
| Ta.3696.2.S1_x_at | -3.901456814 | 7.58E-17 | -3.142516274 | 4.25E-12 | -3.716318504 | 9.27E-16 | -2.901671935 | 1.36E-10 |
| Ta.24544.1.S1_at | -3.928858134 | 4.62E-06 | -3.411947801 | 9.72E-05 | -3.878394968 | 5.69E-06 | -2.723558706 | 0.00305 |
| Ta.23066.2.S1_s_at | -3.93246554 | 1.13E-08 | -2.888895025 | 3.99E-05 | -4.245006881 | 7.69E-10 | -3.063306688 | 1.29E-05 |
| Ta.23032.2.S1_a_at | -3.967949756 | 2.24E-13 | -4.02719488 | 1.27E-13 | -4.277818588 | 4.48E-15 | -3.721059476 | 5.33E-12 |
| Ta.28942.2.A1_at | -3.973663686 | 2.29E-20 | -3.151021469 | 1.70E-14 | -3.771956851 | 5.45E-19 | -3.185375378 | 9.92E-15 |
| Ta.1177.1.S1_at | -4.066949465 | 5.98E-16 | -3.402632156 | 4.09E-12 | -3.951556925 | 2.30E-15 | -3.011015894 | 6.62E-10 |
| Ta.28394.1.S1_s_at | -4.105539718 | 1.50E-13 | -3.140168051 | 8.52E-09 | -4.137286097 | 8.76E-14 | -2.414023061 | 1.27E-05 |
| TaAffx.53723.2.S1_x_at | -4.118938694 | 6.58E-12 | -3.701408425 | 5.78E-10 | -4.13727009 | 4.82E-12 | -3.26602824 | 4.47E-08 |
| Ta.24155.2.S1_s_at | -4.143668704 | 3.74E-40 | -2.297053155 | 2.14E-19 | -4.081994113 | 1.48E-39 | -2.41440582 | 9.40E-21 |
| Ta.16407.1.S1_at | -4.242530188 | 6.66E-17 | -4.589538042 | 6.40E-19 | -4.68273686 | 1.52E-19 | -4.500816577 | 2.34E-18 |
| Ta.20509.1.S1_x_at | -4.381284091 | 3.02E-11 | -4.026132513 | 1.03E-09 | -4.64310871 | 2.38E-12 | -3.747115126 | 1.36E-08 |
| Ta.28750.1.S1_x_at | -4.420631259 | 5.73E-10 | -4.268823266 | 2.47E-09 | -4.684620479 | 5.82E-11 | -4.51330989 | 3.63E-10 |
| Ta.28207.1.S1_at | -4.435829841 | 1.85E-14 | -4.255759454 | 1.62E-13 | -4.691098733 | 8.77E-16 | -3.93977773 | 6.16E-12 |
| Ta.24630.2.S1_x_at | -4.46014757 | 1.46E-13 | -3.909938996 | 5.66E-11 | -4.788037643 | 3.53E-15 | -3.395430808 | 1.12E-08 |
| Ta.28750.2.A1_x_at | -4.58685217 | 6.69E-09 | -4.740356322 | 2.59E-09 | -4.738015902 | 2.07E-09 | -5.118310397 | 1.71E-10 |
| Ta.28750.1.S1_at | -4.614100126 | 6.14E-11 | -4.558663511 | 1.27E-10 | -4.709763346 | 2.54E-11 | -4.565291637 | 1.34E-10 |
| Ta.28394.3.S1_x_at | -4.618927587 | 1.05E-17 | -3.67460182 | 1.59E-12 | -4.891859749 | 2.76E-19 | -2.969931432 | 7.80E-09 |
| Ta.5539.1.S1_at | -4.638702971 | 1.68E-20 | -4.301308917 | 1.98E-18 | -4.67456529 | 8.89E-21 | -4.087311351 | 4.83E-17 |
| Ta.28271.1.S1_at | -4.668104841 | 3.10E-21 | -3.841834787 | 5.29E-16 | -4.912778951 | 8.46E-23 | -3.291123304 | 1.25E-12 |
| Ta.9000.1.S1_at | -4.713131854 | 5.09E-12 | -4.378758908 | 1.27E-10 | -4.288294982 | 2.13E-10 | -4.324380944 | 2.25E-10 |
| Ta.3696.3.S1_a_at | -4.775936315 | 3.41E-23 | -3.647989129 | 8.54E-16 | -4.729205356 | 6.22E-23 | -3.712500457 | 3.44E-16 |
| Ta.23066.1.S1_s_at | -5.254961734 | 4.49E-12 | -4.659777207 | 6.75E-10 | -5.524358185 | 3.95E-13 | -4.249036356 | 1.81E-08 |
| Ta.14729.1.S1_at | -5.337170353 | 7.65E-15 | -5.334526583 | 9.36E-15 | -5.88695272 | 3.49E-17 | -4.965186791 | 3.28E-13 |
| Ta.5539.3.A1_x_at | -5.697321809 | 7.05E-27 | -5.328084071 | 8.98E-25 | -5.68746246 | 6.09E-27 | -5.343121779 | 8.69E-25 |

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