Molecular tools for functional genomic analyses of the stealth pathogenesis of wheat by *Zymoseptoria tritici*

Submitted by Yaadwinder Singh Sidhu to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological sciences,

November 2015

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I certify that all the material in thesis which is not my own work has been identified and that no material has been previously submitted and approved for the award of a degree by this or any other University.

Signature
Abstract

*Zymoseptoria tritici* is an ascomycete fungus that causes *Septoria tritici* leaf blotch disease, which is one of the most devastating diseases of wheat. The lack of molecular tools has withheld functional genomics and consequently has left extensive gaps in the knowledge of the biology of infection by *Z. tritici*. The current research was conducted to develop molecular tools in order to facilitate forward and reserves genetic screens in *Z. tritici*. These tools include an optimised genetic manipulation protocol, the *Z. tritici* strains that provide high frequency targeted genome manipulations, a strategy for gene overexpression and protein tagging, and regulatable promoters for controlled gene expression in *Z. tritici*. The regulatable promoters served to reveal that the *Z. tritici* β-(1,3)-glucan synthase (*BGS1*) gene encoded an essential protein, which regulated cell wall stress tolerance and was therefore, a potential drug target. In addition, these molecular tools revealed a virulence-associated role of the glyoxylate cycle in *Z. tritici* as inactivation of this pathway impeded pre-penetration morphogenesis, which was restored by exogenous glucose application. This result implied that *Z. tritici* engaged the glyoxylate cycle to produce energy through gluconeogenesis by channelling the by-products of lipolysis. This significance of the glyoxylate cycle during initiation of the bi-phasic infection cycle suggests that *Z. tritici* is not a hemibiotroph, but a necrotrophic pathogen with an extended asymptomatic phase of infection. Overall, the molecular tools developed in this study will facilitate large-scale functional genomic analyses to interrogate the biology of infection by *Z. tritici*. The resulting data will inform the development of durable control strategies to combat *Z. tritici* outbreaks.
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Acknowledgements

I would like to thank Biotechnology and Biological Sciences Research Council (BBSRC) and Syngenta UK for the research funding. I am grateful to my supervisors/mentors Professor Ken Haynes and Dr. Michael Csukai for giving me this outstanding career development opportunity and scientific advice. I want to acknowledge the support and kindness of all current and former members of Haynes research group. Equally, I also appreciate Mrs. Eileen Scott, Mrs. Helen Clake, Dr. Sian Deller and Dr. Helen Carter for teaching me basics of fungal biology and their generous support during my research secondment at the Jealotts Hill Research Centre.

I would also like to thank all the members of the Halpin Laboratory including Professor Nicholas J Talbot, Dr Michael Kershaw, Dr. George Littlejohn and Dr. Darren Soanes for their technical support. I acknowledge Dr Sreedhar Kilaru for guidance on various aspect of molecular biology. I want to thank Dr. David Studholme and Dr. Mike Deeks for their advice with bioinformatics and microscopy, respectively. Equally, I want to thank Dr. Marc-Henri Lebrun (BIOGER INRA, France), Dr. Jason Rudd (Rothamsted Research, UK), Dr. Gert Kema (Wageningen University, Netherlands) and their group members for their cooperation throughout my PhD.

Finally, I am extremely grateful and indebted to my family who blessed me with their love and support throughout my PhD. I am fortunate to have very good friends in Yogesh, Neha and others whose company and friendship made it easy to remain upbeat through the peaks and troughs over last four years.
### Abbreviations

<table>
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<th>Description</th>
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<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume ratio</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume ratio</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>µL</td>
<td>Microlitre</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
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<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometre</td>
</tr>
<tr>
<td>nm</td>
<td>Nano meter</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>°C</td>
<td>Degree celsius</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>Mb</td>
<td>Mega base pairs</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilo base pairs</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>DAI</td>
<td>Days after inoculation</td>
</tr>
<tr>
<td>HAI</td>
<td>Hours after inoculation</td>
</tr>
<tr>
<td>MPa</td>
<td>Megapascals</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>ms</td>
<td>Millisecond</td>
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Chapter 1

General Introduction
1.1 Global importance of plant pathogens

Since the dawn of agriculture, plant pathogens have been a major constraint on food production and severe crop disease epidemics have caused large-scale human suffering (Oerke, 2006, Bebber et al., 2013, Strange and Scott, 2005). For example, over one million human lives were claimed by the 1845 Irish potato famine caused by the oomycete pathogen *Phytophthora infestans* and the 1943 Great Bengal famine caused by the fungus *Helminthosporium oryzae* resulted in death of more than two million people (Fisher et al., 2012, Strange and Scott, 2005). In addition to the loss of human lives, the aftermath of these two epidemics saw mass migration and left lasting geopolitical changes in Europe, Southeast Asia and the Americas. Alongside agriculture, recent epidemics in forestry including ash dieback and Dutch elm disease caused by the fungal pathogens *Hymenoscyphus fraxineus* and *Ophiostoma ulmi*, respectively, have threatened the ecological biodiversity throughout the natural landscapes in Europe (Forestry-Commission, 2015, Fisher et al., 2012).

Agricultural crops routinely face several abiotic and biotic stresses. Abiotic stresses, such as water and nutrient deficiency can be supplemented through irrigation and fertiliser application; however, the losses incurred from biotic stresses, particularly disease outbreaks, are often much more difficult to mitigate (Oerke, 2006). Annually around 10% of the global food crop yield is lost to disease (Strange and Scott, 2005). Between 2001 and 2003, bacterial and fungal diseases are estimated to have claimed around 10% (equivalent to 94 million metric tonnes) of the globally attainable yield of wheat (*Triticum* species) (Oerke, 2006). These losses are likely to increase in the future as global climate
change could intensify disease incidence (Bebber et al., 2013). Furthermore, elevated temperatures will negatively influence the crop physiology and consequently reduce crop yields (Peng et al., 2004). Evidence shows that crop yield losses due to disease outbreaks are a major hurdle in increasing global food production (Fisher et al., 2012, Oerke, 2006). Therefore, the projected rise in food demands due to increasing human population could be partially met by minimising crop losses to disease (Oerke, 2006). For example, the global yield of five major staple food crops (namely wheat, rice, maize, potato and soybean) that was lost to disease between 2009 to 2010 was sufficient to provide annual nutrition (2000 kcal day\(^{-1}\)) to over 4 billion people (Fisher et al., 2012). Therefore, it is essential to gain a comprehensive understanding of the biology of host-pathogen interactions in order to develop sustainable and durable disease control measures.

1.2 An overview of microbial pathogenesis and plant defences

Before plant pathogens can initiate disease, they have to overcome the pre-formed host defences known as the passive or non-host resistance. These passive defences include the outermost waxy surface layer known as the cuticle, and plethora of antimicrobial metabolites (Dangl and Jones, 2001). Plant pathogens have evolved diverse strategies to overcome the non-host resistance. Some pathogens such as the rice blast causing fungus Magnaporthe oryzae directly overcomes the passive defences in rice (Oryza sativa) by mechanically rupturing the cuticle (Wilson and Talbot, 2009, Tucker and Talbot, 2001). While some other pathogens such as Z. tritici, which causes the Septoria tritici leaf blotch (STB) disease in wheat, exclusively exploits the stomata to invade wheat leaves in order to initiate disease (Kema et al., 1996).
Once inside the host, pathogens face a plethora of the toxic metabolites, such as saponins including avenacin (a tri-terpene saponin produced by wheat) that effectively halt microbial growth (Mysore and Ryu, 2004). Host-specialised pathogens detoxify these antimicrobial compounds by producing various enzymes such as the avenacin hydrolysing β-D-glucosidase secreted by the fungus *Gaeumannomyces graminis var. tritici*, which causes take all in wheat and oats (*Avena sativa*) (Wubben et al., 1996).

**Figure 1.1 The zigzag model of plant defence response.**

Pathogens are detected in plants through conserved pathogen-associated molecular patterns (PAMPs) (red diamonds) leading to induction of PAMP-triggered immunity (PTI). Pathogens secrete effectors (red circles) to suppress PTI, which leads to effector-triggered susceptibility (ETS). Subsequently, effector recognition leads to effector-triggered immunity (ETI), which is a stronger defense response and surpasses the threshold of hypersensitive response (HR). Pathogens evolve to loose ETI-triggering effectors (red circle) and/or gain new ETI-suppressing effectors (blue circles). In parallel, plants evolve to recognize these new ETI-suppressing effectors to maintain elicitation of ETI. Figure and legend adapted from Jones and Dangl (2006).

After compromising the passive defences, pathogens face multiple layers of the inducible innate host defences, which are represented as the zig-zag model (Figure 1.1) (Mysore and Ryu, 2004, Dangl and Jones, 2001, Jones and
Dangl, 2006). These innate defences are triggered by pathogen associated molecular patterns (PAMPs), which are pathogen-related conserved signatures such as the bacterial flagellin and fungal cell wall polysaccharides (Hayashi et al., 2001, Latgé, 2010, Dangl et al., 2013, Jones and Dangl, 2006, Dangl and Jones, 2001). Plants recognise PAMPs through the pattern recognition receptors (PRRs), which elicit the first phase of innate defence response known as the PAMP triggered immunity (PTI) (Jones and Dangl, 2006, Dodds and Rathjen, 2010). The PTI is a medium amplitude defence response, which comprises accumulation of reaction oxygen species (ROS), hydrolytic enzymes and low molecular weight antimicrobial compounds known as phytoalexins, such as camalexin (an indole phytoalexin) (Mysore and Ryu, 2004).

Pathogens overcome PTI by secreting effector molecules, which can either be toxic metabolites or proteins that kill the host plant (Lo Presti et al., 2015). In addition, the secreted proteins that safeguard the fungus by suppressing host immunity of manipulating host physiology are also regarded as effectors (Lo Presti et al., 2015). Typically the secreted effector proteins are characterised by cysteine rich content, small size and extracellular secretion sequence and are termed as small-secreted proteins (SSPs) (do Amaral et al., 2012, Mirzadi Gohari et al., 2015, Vleeshouwers and Oliver, 2014). In parallel to PTI suppression and subsequent effector triggered susceptibility (ETS), the effectors get recognised through direct or indirect interactions with the host resistance (R) proteins (Jones and Dangl, 2006, Dodds and Rathjen, 2010). The R proteins are characterised by the nucleotide binding (NB) and leucine rich repeat (LRR) domains and are termed as the NB-LRR proteins (Jones and Dangl, 2006, Dodds and Rathjen, 2010). As a consequence of detection of effectors, the ETS is followed by induction of the second line of defence known
as effector triggered immunity (ETI), which is a much stronger anti microbial defence response as compared to the initial PTI (Figure 1.1)(Jones and Dangl, 2006). Pathogens have evolved to overcome the ETI either by negatively selecting to loose the genotypes containing ETI-triggering effectors and/or positively selecting to gain multiple additional ETI-suppressing effectors, which effectively reinstate ETS. In parallel, evolution of the R protein has enabled recognition of ETI-suppressing effectors and reinforcement of ETI response. As a consequence of ETI, accumulation of ROS leads to host cell death known as the hypersensitive response (HR), which coincides with emergence of typical disease symptoms including necrotic lesions (Jones and Dangl, 2006, Dodds and Rathjen, 2010).

Overall, the compatible plant-pathogen interactions involve highly sophisticated molecular arsenals to attack and defend against each other. As a consequence, plants and their pathogens are co-evolving whilst being engaged in an arms race (Anderson et al., 2010). Moreover, the modern agricultural practices are imposing immense selection pressure on pathogens. Consequently, resistant pathogen genotypes are selected more rapidly than the rate of discovery of new disease control measures. It is therefore crucial to understand plant-pathogen interactions in order to identify novel sources of genetic resistance.

1.3 Global importance of *Mycosphaerella* pathogens

The genus *Mycosphaerella* belongs to the *Mycosphaerellaceae* family in the Ascomycota division of the kingdom Fungi. This genus contains more than three thousand species of plant pathogenic fungi (Quaedvlieg, 2014). *Mycosphaerella* pathogens have evolved diverse life styles. Majority of the
Mycosphaerella species are necrotrophic, biotrophic, or hemibiotrophic pathogens, however a small number have also evolved as symbiotic, endophytic and saprophytic fungi (Quaedvlieg, 2014, Crous et al., 2007). Mycosphaerella pathogens infect a range of plant species and each year these infections cause major economic damages in forestry, horticulture and agriculture. The European and Mediterranean Plant Protection Organization (EPPO) of the European Union (EU) have classified seven Mycosphaerella pathogens among 350 quarantine species, which present the most serious threat to ecosystem and environment (Quaedvlieg, 2014).

Around ninety Mycosphaerella species are primary and secondary pathogens of Eucalyptus (Myrtaceae species) and are a major constraint on Eucalyptus production for global timber industry (Crous et al., 2007). Likewise, Mycosphaerella pinodes, the causative agent of Ascochyta blight of common pea (Pisum sativum), prevails in all the global agroclimatic zones and annually claims up to 30% of the attainable pea yield (Roger et al., 1999). Another horticultural pathogen, M. fijiensis causes the black leaf streak disease or black sigatoka disease, which is the most important disease of banana (Musa species) (Churchill, 2011). Severe M. fijiensis outbreaks can reduce banana production by 80% and annual cost of M. fijiensis control amounts to 30% of the total production cost (Churchill, 2011). M. musicola is another major cause of black sigatoka disease of banana in the sub-Saharan Africa and yield reductions from M. musicola outbreaks are comparable to those incurred by M. fijiensis (Gomes et al., 2013). Similarly, each year a significantly large proportion of attainable vegetable yield is lost to outbreaks of M. brassicicola and M. capsellae, which cause the ring rot of brassicas (Wakeham and Kennedy, 2010, Götz et al., 1993) and white leaf spot of crucifers (Inman et al.,
1991), respectively. *Cladosporium fulvum* is another important *Mycosphaerella* pathogen that causes a devastating disease known as tomato leaf mould. However, *C. fulvum* is no longer a threat to tomato production since the *C. fulvum* resistance gene *Cf*-9 was introduced into commercial tomato cultivars (Thomma et al., 2005).

Despite their economic importance, the biology and epidemiology of most *Mycosphaerella* pathogens is relatively unknown. This is mainly due to difficulties in identification of different *Mycosphaerella* species. Identification is further complicated by poor *in vitro* sporulation, morphologically conserved small fruiting bodies, and occurrence of multiple species within a single disease lesion (Quaedvlieg, 2014). In recent years, study of the *Mycosphaerella* pathogens has benefitted from reducing costs of sequencing technologies and comparative genomics. This has paved the way for elucidation of phylogenetic histories and reclassification of various species including *Z. tritici* and its sister species (Quaedvlieg et al., 2011, Stukenbrock et al., 2012). The genomics revolution and genetic tractability has meant that *C. fulvum* and *Z. tritici* are now two of the most well studied members of *Mycosphaerellaceae*. As result, *C. fulvum* and *Z. tritici* are increasingly proposed as model systems for other *Mycosphaerella* pathogens (Thomma et al., 2005, Goodwin et al., 2004).

1.4 Wheat and *Septoria tritici* leaf blotch

Wheat, a monocot cereal, is the most important food crop in the world and its annual production exceeds that of any other major food crop (including rice, potato and maize) (Curtis, 2002). Globally, wheat is cultivated in over 200 million hectares and as a staple food wheat provides 20-25% of the calories and protein content for over 2.5 billion people (Wheat.org, 2014, Curtis, 2002).
By 2050, the current global wheat production must increase by 60-70% to adequately suffice projected rise in food demands (Wheat.org, 2014). The increase in production needs to be sustainable, without encroaching on more land and while reducing the agrochemical inputs (Wheat.org, 2014). A major challenge in increasing wheat production is presented by several fungal pathogens that infect wheat (Oerke, 2006). Globally, the most damaging fungal diseases of wheat are head blight (caused by *Gibberella zeae*), powdery mildew (caused by *Blumeria graminis*), STB (caused by *Z. tritici*) and stem rust (caused by *Puccinia graminis*) (Dean et al., 2012, Oerke, 2006).

STB is one of the most economically devastating foliar diseases of cultivated wheat landraces namely bread wheat (*Triticum aestivum*) and durum wheat (*Triticum durum*) (Goodwin et al., 2011, Eyal et al., 1987). Typical STB disease symptoms include formation of chlorotic and necrotic spots, and black brown lesions on the surfaces of infected wheat leaves (Figure 1.2 A). Consequently, reduction in the photosynthetically active radiation intercepting green leaf area lowers the net photosynthetic capability of the infected plants. As a result, alteration to photosynthetic energy assimilation processes adversely affects normal growth, especially the development of grain bearing heads, which in turn reduces the wheat yield (Robert et al., 2006). STB and its causative agent *Z. tritici* (previously known as *Mycosphaerella graminicola*) are prevalent throughout all the wheat growing regions of the world (Cools and Fraaije, 2008, Goodwin et al., 2011). Severe STB outbreaks can reduce wheat yields by up to 50% (Eyal et al., 1987, Fones and Gurr, 2015). In Europe, €700 million worth of wheat yield is lost annually to STB (Fones and Gurr, 2015, Orton et al., 2011). A further €500 million is spent on fungicides to control STB outbreaks in Germany, France and the United Kingdom alone (Fones and Gurr,
2015). Similar economic costs of STB outbreaks are also reported in other parts of the world. Therefore, STB outbreaks present a major threat to global wheat production.
Figure 1.2 Growth morphologies of *Z. tritici* and STB disease cycle.

(A) Typical STB disease symptoms including chlorosis, necrosis and pycnidial lesions formed on the surface of infected wheat leaves. (B) *Z. tritici* ascospores germinating on the wheat leaf surface. (C) *Z. tritici* pycnidiospores differentiating into pseudohyphae and spores through lateral budding (arrowheads); lower panel shows calcofluor white stained septa. (Bar = 10 µm). (D) *Z. tritici* pycnidiospores expressing green fluorescent protein and wheat cell wall labelled with red fluorescent stain propidium iodide. (Bar = 25 µm). (E) Elongated filamentous *Z. tritici* hyphae and yeast-like budding spores (arrowheads). (Bar = 10 µm). (F) *Z. tritici* hyphae penetrating and/or growing over the stomata on the wheat leaf surface. (G) STB disease cycle depicting the primary and secondary infection stages of the polycyclic infection process. (Figure 1.2 A, C, D and E taken from Steinberg et al., (2015) and Figures 1.2 B, F and G taken from Ponomarenko et al., (2011)).

1.5 Biology of *Z. tritici* and its mode of pathogenesis

1.5.1 The *Z. tritici* genome

The genome sequence of the *Z. tritici* reference isolate IPO323 is 39.7 Mb, out of which 21% comprises of repetitive nucleotide sequences (Goodwin et al., 2011). The *Z. tritici* genome contains 10933 protein-coding genes dispersed over 21 chromosomes. The majority of the *Z. tritici* genes are present on chromosomes 1 to 13, known as the core chromosomes (CCs) (Goodwin et
Around 700 genes are present on chromosomes 14 to 21, which are known as the accessory chromosomes (ACs) and can be lost or gained during meiosis, individually or in combinations, without any observable fitness penalty to *Z. tritici* (Goodwin et al., 2011, Wittenberg et al., 2009, Kellner et al., 2014). The ACs range from 0.4 to 1 Mb in size and form 12% of the *Z. tritici* genome (Grandaubert et al., 2015, Goodwin et al., 2011). The ACs contain half the gene density and double the repeat element content as compared to the CCs (Grandaubert et al., 2015, Schotanus et al., 2015). Most genes on the ACs lack annotation and remain transcriptionally silent and were expressed at relatively lower levels during *in vitro* growth and *in planta* infection (Kellner et al., 2014, Yang et al., 2013a). On average, the expression of the genes on ACs is 13 fold lower as compared to those on the CCs (Kellner et al., 2014). This variation in gene expression is due to differences in the chromatin organization and histone modifications between the CCs and ACs (Schotanus et al., 2015). It is speculated that these chromatin modifications serve as a transcriptional regulatory mechanisms (Schotanus et al., 2015) in absence of cytosine DNA methylation mediated epigenetic regulation of gene expression in *Z. tritici* (Dhillon et al., 2010, Goodwin et al., 2011).

The ACs are one of the most fascinating aspect of the *Z. tritici* genome biology despite their enigmatic functional significance. The majority of genes on ACs are unique and not paralogs of those on the CCs (Kellner et al., 2014, Grandaubert et al., 2015) which suggests that the ACs originated from complex structural genomic rearrangements (Schotanus et al., 2015) and not from simple evolutionary duplication or degeneration of the CCs (Goodwin et al., 2011). Similar “dispensable” ACs with partially homologous nucleotide
sequence are also found in close relatives of Z. tritici (Schotanus et al., 2015). This points towards an ancestral origin of the ACs and indicates that their evolutionary conservation in Z. tritici may offer some functional advantages (Schotanus et al., 2015). The advantages of such genomic plasticity and the ACs are further strengthened by the fact that not a single Z. tritici isolate has been discovered to lose all the eight ACs (Perez-Nadales et al., 2014). Genes on the ACs evolve more rapidly than those on CCs (Croll and McDonald, 2012) and this unrestricted genome evolution could be advantageous for rapid development of virulence related genes in Z. tritici (Stukenbrock et al., 2010, Schotanus et al., 2015, Poppe et al., 2015). Recent genomics studies have made some headway in deciphering the origins of the Z. tritici ACs and future research will play a key role in providing new insights into the virulence-related role of genome plasticity in Z. tritici.

1.5.2 Taxonomy and growth morphologies

Z. tritici is a heterothallic ascomycete fungus that belongs to the genus Zymoseptoria of the Mycosphaerellaceae family in the class Dothidiomycetes. Phylogenetic history revealed that Z. tritici emerged as a host specific pathogen alongside the domestication of wheat in the Fertile Crescent (modern day Iraq, Syria and Lebanon) (Stukenbrock et al., 2007). In the field, Z. tritici populations are a mixture of two mating types, MAT1-1 and MAT1-2 (Kema et al., 1996) and the sexual reproduction between these mating types leads to formation of ascospores (teleomorph) (Figure 1.2 B) (Suffert et al., 2011). The link between ascospores and the STB disease was first described in 1842 (Desmazières, 1842), while the role of asexual pycnidiospores (anamorph) (Figure 1.2 C D) in STB infection was reported much later in 1972 (Sanderson, 1972). Highly
efficient sexual and asexual reproduction in *Z. tritici* leads to heterogenetic and a diverse population structure (Zhan et al., 2003). The asexual reproductive cycle allows *Z. tritici* to rapidly propagate fitness traits such as resistant genotypes, while the sexual reproduction allows for rapid gene flow to facilitate the spread of such traits over large geographical areas (Orton et al., 2011). This heterothallic nature makes *Z. tritici* a successful pathogen, which is highly challenging in terms of durable disease control (Talbot, 2015).

The *in vitro* and *in vivo* growth of *Z. tritici* is polymorphic and heterogeneous (Figure 1.2 C and E) (Steinberg, 2015, Eyal et al., 1987). *Z. tritici* differentiates into unicellular and multicellular spores known as micro- and macro-pycnidiospores, respectively. The *In vitro* grown *Z. tritici* spores are also termed as “budding or yeast-like-propagation” (Motteram et al., 2009, Goodwin et al., 2011, Steinberg, 2015) and are widely regarded as a laboratory artefact, which is believed to be absent from the STB infection cycle (Eyal et al., 1987). However, the bacillus-shaped microspores resembling the *in vitro* grown spores were found in the pycnidia form on infected leaf surfaces (Suffert et al., 2011). Nutrient deficiency and elevated temperatures (typically around 25 °C) trigger polar tip elongation in the *Z. tritici* spores, leading to the formation of filamentous hyphae (Figure 1.2 E) (Motteram et al., 2009, Steinberg, 2015, Kema et al., 1996). These *in vitro* grown hyphae resemble the *Z. tritici* hyphae found on wheat leaves (Figure 1.2 E and F), which are a prerequisite for the initiation of STB disease (Mehrabi et al., 2006b, Kema et al., 1996, Steinberg, 2015).

### 1.5.3 STB disease cycle

#### 1.5.3.1 Disease initiation
Z. tritici ascospores are the primary inoculum for disease initiation. Ascospores are released from the overwintered pseudothecia (or perithecia) and dispersed by wind currents over large geographical areas (Figure 1.2 G) (Brown et al., 1978, Suffert et al., 2011, Eyal et al., 1987). The pycnidiospores emerge at the end of the first cycle of infection (Figure 1.2 D and G) and these secondary inoculums are dispersed by rain splash during multiple cycles of infection. High humidity, rainfall and temperatures are crucial factors that modulate the release of ascospores and pycnidiospores (Eyal et al., 1987, Suffert et al., 2011).

The Z. tritici infectious propagules must adhere to the hydrophobic surface of the wheat leaves in order to initiate STB disease cycle. However, the mechanisms of adherence adopted by Z. tritici remain unknown. Future research into the biological pathways involved in attachment of Z. tritici spores to wheat leaves could reveal novel druggable targets for the development of fungicides for early stage disease intervention. For instance, the conidia of the rice blast fungus M. oryzae contain large apical deposits of the spore-tip-mucilage, which facilitates attachment and is secreted following hydration upon contact with the rice leaves (Hamer et al., 1988). The secretion of spore-tip-mucilage by M. oryzae can be inhibited using a type of lectin known as concanavalin A (Hamer et al., 1988).

The secreted hydrophobin proteins are well known for their role in adherence of fungal pathogens (Perez-Nadales et al., 2014, Amanianda et al., 2009, Talbot et al., 1993). It is possible that Z. tritici hydrophobins could be involved in adherence to wheat leaves. Indeed the transcriptional data (Rudd et al., 2015, Yang et al., 2013a) shows that four Z. tritici class II hydrophobin
coding genes namely Mycgr3G48129, Mycgr3G40724 Mycgr3G96543 and Mycgr3G95491 were highly up regulated at early stages of infection. This expression pattern of Z. tritici hydrophobin is similar to M. oryzae in which six hydrophobin showed elevated expression during the early phase of infection (Kawahara et al., 2012). One of these, the M. oryzae gene MPG1 encodes a hydrophobin protein that is critical for the spread of rice blast disease (Talbot et al., 1993). The expression of the M. oryzae MPG1 was elevated in carbon and nitrogen poor conditions and the Z. tritici ortholog encoded by gene model Mycgr3G48129 also showed the same elevated expression under these conditions (Rudd et al., 2015). The early stage up regulation suggests that of the Z. tritici hydrophobins could be facilitating attachment to wheat leaf surfaces.

1.5.3.2 Host penetration

Z. tritici ascospores and pycnidiospores rapidly sense the leaf environment and develop into infectious hyphae (Figure 1.2 F G) within 12-24 hours of arriving on wheat leaves (Suffert et al., 2011, Shetty et al., 2003, Ponomarenko et al., 2011). This rapid detection and adaptation to the host environment is evident from differential expression of more than 1000 Z. tritici genes within 24 hours after infection (HAI), out of which around 200 genes are shown to have peak expression at this early stage (Rudd et al., 2015). The spore-hyphal morphogenesis is modulated by mitogen-activated protein kinase (MAPK) proteins HOG1, FUS3 and SLT2 and cyclic adenosine monophosphate (cAMP) signalling pathway (Mehrabi et al., 2006a, Mehrabi and Kema, 2006, Mehrabi et al., 2009, Cousin et al., 2006, Mehrabi, 2006, Kramer et al., 2009). Mutants lacking the genes encoding for these MAPK proteins and the cAMP
regulatory protein-coding gene GPB1 were unable to differentiate into infectious hyphae, which led to attenuation and/or severe reduction in virulence (Mehrabi et al., 2009). The MAPKs and cAMP signalling pathways are essential for infection related morphogenesis in a range of pathogenic fungi (Wilson and Talbot, 2009), which suggests that these global regulators play a widely conserved role in fungal virulence.

*Z. tritici* hyphae are the sole known invasive morphology and these invade wheat leaves exclusively through the stomata (Rudd, 2015, Steinberg, 2015). The consensus is that *Z. tritici* does not engage in direct penetration of the host through specialised structures such as the appressoria formed by *M. oryzae* (Dagdas et al., 2012); although, direct penetration by *Z. tritici* hyphae and formation of swollen appressorium like structures at the hyphal tips has been occasionally reported (Kema et al., 1996, Cohen and Eyal, 1993, Shetty et al., 2003). Absence of direct host invasion by *Z. tritici* is also plausible from the fact that its genome contains only 28 genes that encode plant cell wall degrading enzymes (PCWDEs), which is a much smaller number as compared to other plant pathogens (Goodwin et al., 2011). Furthermore, majority of the PCWDE coding genes are expressed when switching from the asymptomatic to symptomatic phase or later (Brunner et al., 2013). The digestion of host cell wall by PCWDEs releases polysaccharides, which act as PAMPs (Vorwerk et al., 2004) and trigger PTI (Boyd et al., 2013). Thus, it appears that *Z. tritici* has adapted to exclusive stomatal penetration and fine-tuned expression of the PCWDE coding genes to evade immunodetection and minimise PTI response.

*Z. tritici* hyphae enter into the stomata at random and the chemo- or thigmo-tropic signals are believed to be absent (Kema et al., 1996, Steinberg,
This is despite a study that reported presence of an unknown thigmotropic signal, which directed hyphal elongation towards the stomata (Duncan and Howard, 2000). Often the hyphae swell up upon entry into stomata (Kema et al., 1996, Siah et al., 2010, Orton et al., 2011), however the mechanisms, which trigger this swelling and its role in stomatal penetration remains unknown.

1.5.3.3 Host colonisation

Once inside wheat leaves, the hyphae continue extracellular growth with respect to host cells for an extended period without any macroscopic superficial symptoms of STB disease (Rudd et al., 2015, Kema et al., 1996). This asymptomatic phase from the arrival of the fungus on the leaf, to the appearance of visible symptoms, is referred to as the latent period (Rudd, 2015, Lovell et al., 2004). This phase of infection typically ranges from 8 to 14 days, and in some cases even 42 days, depending on climatic conditions and wheat cultivars (Lovell et al., 2004). Controversially, the asymptomatic phase is also referred to as the biotrophic phase of the “hemibiotrophic” lifestyle of Z. tritici despite the fact that fungus does not engage in biotrophic feeding and nor does it differentiate the biotrophy associated specialised feeding structures such as haustoria (Orton et al., 2011).

During asymptomatic phase, the Z. tritici growth is slow and this leads to a relatively small increase in fungal biomass. Z. tritici remains restricted to nutrient deficient apoplastic cavities within the mesophyll layer (Kema et al., 1996, Duncan and Howard, 2000). Z. tritici spores are rich in lipids and fatty acids, which are thought to provide the nutrition and energy for growth during disease initiation and asymptomatic phase of infection (Rudd et al., 2015). This
notion is strengthened by the early stage (between 24 and 96 HAI) up regulation of the *Z. tritici* genes encoding proteins that function in lipid and fatty acid transport and catabolism through β-oxidation and the glyoxylate cycle (Rudd et al., 2015, Yang and Yin, 2015). Stored lipids and fatty acid reserves are key source of energy in several necrotrophic fungal pathogens such as *Stagonospora nodorum* and *Leptophaeria maculans*, which cause wheat blotch and blackleg disease in *Brassica* species, respectively (Solomon et al., 2004, Idnurm and Howlett, 2002). Therefore, the early stage up regulation of lipid metabolism in *Z. tritici* implies that consistent with other necrotrophic pathogens this fungus may also be utilising stored lipid reserves to produce energy for sustaining minimal fungal growth during the early stages of STB infection.

**1.5.3.4 Host immune response**

The full extent of host response during asymptomatic phase is relatively unknown, however fungal presence does trigger host immunity. The host immunity is initiated within 48 HAI as evident from chloroplast accumulation near the host cell walls and in contact with the *Z. tritici* hyphae (Kema et al., 1996). Between 72 and 120 HAI the hydrogen peroxide (one of the reactive oxygen species (ROS) associated with the plant defences) accumulates in the vicinity of *Z. tritici* hyphae within host cell walls and within the sub-stomatal cavities (Shetty et al., 2003). In addition, the detection of *Z. tritici* in wheat leads to elevated expression of the wheat genes encoding for pathogen resistance (PR) proteins (Ray et al., 2003) and fungal cell wall degrading enzymes (Shetty et al., 2007, Shetty et al., 2003, Shetty et al., 2009).

Although the mechanisms that modulate immunodetection of *Z. tritici* are currently unknown, It highly likely that fungal cell wall polysaccharides,
especially glucan and chitin, which are also key PAMPs that elicit PTI (Latgé, 2010), play a key role in detection of *Z. tritici* by wheat. Indeed the β-(1,3)-glucan isolated from the *Z. tritici* cell wall has been shown to mediate PTI response during early stages of STB infection (Shetty et al., 2007, Shetty et al., 2009). Although PTI is rapidly induced, the extent to which this defence response safeguards wheat against invasion by *Z. tritici* remains unclear (O’Driscoll et al., 2014).

1.5.3.5 Suppression of host immune response by *Z. tritici*

*Z. tritici* continues to survive and develop throughout to the asymptomatic phase of infection, which implies that this fungus is able to overcome or suppress PTI response in wheat. The suppression of PTI by *Z. tritici* is evident from reduced expression of the wheat genes that encode for biotic defence related proteins including the PR proteins, WRKY transcription factors, MAPKs and receptor kinases, and proteins involved in biosynthesis of defence signalling molecules Jasmonic acid and Oxylin, and lignin biosynthesis (Rudd et al., 2015).

Fungal pathogens secrete effector molecules to suppress PTI (Boyd et al., 2013). *Z. tritici* has evolved an extensive repertoire of small secreted proteins (SSPs) which may also function as effectors (Mirzadi Gohari et al., 2015, do Amaral et al., 2012). Most of the *Z. tritici* SSP coding genes are expressed in successive waves to correspond with typical stages of the bi-phasic infection cycle (Mirzadi Gohari et al., 2015). Such waves of effector expression are common in fungal pathogens which engage transition from asymptomatic to symptomatic phases, such as *Colletotrichum higginsianum* and *Colletotrichum graminicola*, which infect *Arabidopsis thaliana* and maize (*Zea mays*),
respectively (O’Connell et al., 2012, Mirzadi Gohari et al., 2015). These waves of gene expression indicate that Z. tritici fine-tunes expression of putative effectors to counter the stage specific activation of host defences (Rudd et al., 2015).

Around 38% (366/970) of the Z. tritici SSP coding genes were differentially expressed at various stages of the infection cycle and 16% (155/970) showed peak expression within 24 HAI (Rudd et al., 2015). This expression of the Z. tritici SSPs is similar to M. oryzae, which suppresses PTI by secreting effectors (Mentlak et al., 2012) and showed early stage up regulation of more than two hundred SSP coding genes (Kawahara et al., 2012). Although a large number of SSPs have been suggested as putative effectors, however, only one Z. tritici effector, denoted as Mg3LysM, has been cloned to date (Marshall et al., 2011). The Z. tritici Mg3LysM suppresses PTI by interfering with the wheat CERK1 receptor kinase and the CEBiP receptor-like proteins, which serve to detect this pathogen by recognising chitin, an important fungal cell wall component (Lee et al., 2014). The Z. tritici Mg3LysM is an ortholog of the M. oryzae effector Slp1, which is highly expressed during infection (Kawahara et al., 2012) to suppress the PTI response in rice and to enable spread of the rice blast disease (Mentlak et al., 2012).

Effectors are emerging as an important tool for identification of novel sources of genetic resistance for breeding new disease resistant cultivars (Vleeshouwers and Oliver, 2014). As a result large proportion of research is focused on cloning novel Z. tritici effectors that underpin the compatible and incompatible outcomes in the Wheat- Z. tritici pathosystem (Rudd et al., 2015, Mirzadi Gohari et al., 2015). For example, early stage (around 96 HAI) up
regulation of several chloroperoxidase-coding genes suggested that their products could be active against ROS produced by wheat (Rudd et al., 2015). However, due to dispensability of various SSPs, the induced expression of these SSP coding genes may not be enough to verify their role in virulence of *Z. tritici*. Indeed the targeted deletion of the several SSP coding genes, which displayed highly elevated expression at various stages of infection, failed to cause any detectable defects in virulence of the *Z. tritici* mutants (Mirzadi Gohari et al., 2015, Rudd et al., 2015). Unlike the MAPK proteins, orthologs of which play widely conserved role even in distantly related fungi, the high polymorphism between effectors implies that known effectors which determine virulence in one species could be dispensable in another. For example, the secreted necrosis and ethylene-inducing peptide like proteins (NLPs) are required by various prokaryotic and eukaryotic plant pathogens to promote host cell death and subsequently necrotrophic pathogen growth (Albert et al., 2015), however the *Z. tritici* MgNLP was not required for normal fungal virulence (Motteram et al., 2009). Due to the functional redundancy, it is possible that majority of the *Z. tritici* SSPs will be dispensable for its virulence on wheat. This functional redundancy is a major bottleneck in cloning novel *Z. tritici* effectors and as result has withheld identification of novel sources of durable genetic resistance in wheat.

### 1.5.3.6 Transition to the symptomatic phase of infection

The symptomatic phase (also known as the necrotrophic phase) follows on the asymptomatic phase (Rudd, 2015). This leads to the emergence of typical STB disease symptoms, which include macroscopic chlorotic and necrotic lesions (around 8 to 14 days after infection (DAI)) and the formation of
black brown pycnidial lesions (around 12 to 15 DAI) on the surface of infected leaves (Figure 1.2 A). The molecular mechanisms that signal for this phase transition are relatively unknown, however, it is evident that host cell death plays a major role in the switch from asymptomatic to symptomatic phase. The host cell death is mediated through a programmed-cell-death (PCD) like response leading to leakage of the host cytosolic components into the apoplastic cavities (Keon et al., 2007). In addition, the ROS accumulation and activation of wheat defences by homeodomain protein TaR1 are also essential for normal switch to symptomatic phase of infection (O'Driscoll et al., 2014, Keon et al., 2007, Lee et al., 2015). The down regulation of the wheat TaR1 gene, through gene silencing, impaired the transcriptional activation of the wheat genes with open chromatin and lead to early appearance of STB disease symptoms and reduced fungal sporulation (Lee et al., 2015). In addition to the host cell death and the Tar1 protein, the switch to the symptomatic phase also overlapped with up and down regulation of several wheat genes which encode proteins involved in biotic stress resistance, transport, metabolism, signal transduction and photosynthesis (Yang et al., 2013a, Rudd et al., 2015). Overall, it appears that major molecular reprogramming of host defence plays a key role in modulating the switch from the asymptomatic to symptomatic phase of STB infection.

In *Z. tritici*, switch to the symptomatic phase coincided with the biggest wave of gene expression involving peak expression of more than 24% (235/970) SSP coding genes (Rudd et al., 2015). It is likely that many of these may serve to combat the extensive host defence response, although targeted deletion of five SSP coding genes did not influence virulence of *Z. tritici* (Rudd et al., 2015). Interestingly, the *Z. tritici* genes *Mg1LysM* and *Mg3LysM*, which
were initially through to be expressed only during the asymptomatic phase (Marshall et al., 2011), were also up regulated during the asymptomatic phase (Yang et al., 2013). Therefore, it is likely that the protective role of \textit{Z. tritici} Mg1LysM and Mg3LysM against the host secreted chitinases stretches beyond the asymptomatic phase of STB infection (Yang et al., 2013).

Plants are well known to engage in PCD response upon ROS production in order to control the spread of a pathogen (Gadjev et al., 2008); however, in the Wheat- \textit{Z. tritici} pathosystem, the fungal biomass exponentially increases following host cell death (Keon et al., 2007, Shetty et al., 2007, Rudd, 2015). Thus, \textit{Z. tritici} undermines the PCD mediated host defences to fuel the increase in fungal biomass possibly by utilising leaked host cytosolic contents including several polysaccharides (Shetty et al., 2009, Rudd, 2015). The increase in fungal biomass further escalates the host tissue collapse, which coincides with maturation of the pycnidia and formation of secondary inoculum, the asexual pycnidiospores. The rains during the wheat-growing season cause humid conditions within the crop canopy and leads to pycnidiospores release and dispersal to neighbouring leaves to reinitiate multiple cycles of the STB disease.

In summary the bi-phasic infection cycle of \textit{Z tritici} resembles that of the necrotrophic fungi such as \textit{L. maculans} (Idnurm and Howlett, 2002), which sustain minimal growth for an extended period within the host by utilising internal nutrient reserves without relying on biotrophic feeding, and switch to the symptomatic phase coincides with extensive fungal growth (Sanchez-Vallet et al., 2015). Therefore, it appears that \textit{Z. tritici} is not a hemibiotrophic but a necrotrophic fungal pathogen with extended latent or asymptomatic phase of infection (Rudd, 2015). This trophic nature of \textit{Z. tritici} is plausible as lengthy
asymptomatic phases of are also reported for various other necrotrophic pathogens in the *Mycosphaerellaceae* family (Rudd, 2015).

It is evident that several questions regarding the molecular mechanisms, which underlie the compatible Wheat- *Z. tritici* interactions, remain unanswered. Questions regarding the state of dormancy and adherence of the infectious propagules require further investigations. In parallel, it is important to investigate what enables *Z. tritici* to grow, evade and/or overcome the host immune response during the asymptomatic phase? Equally, the questions about the particular molecular cross talks between *Z. tritici* and wheat that initiate the phase transition during infection can also not be ignored. Similarly, the question about nutrition sources and assimilation mechanisms that enable the fungus to fuel pre-penetration growth and exponential biomass increase during symptomatic phase also need to be studied. The answers to these fundamental questions are crucial in order to understand the mechanisms that underpin the stealth mode of pathogenesis adopted by *Z. tritici*. Consequently, a comprehensive biological understanding of the disease cycle will inform development of durable STB control measures.

1.6 STB disease control

STB disease control mainly relies on fungicides and resistant wheat cultivars. Fungicides are the dominant strategy for combating STB outbreaks (O'Driscoll et al., 2014, Torriani et al., 2015); however, rapid evolution of fungicide resistance implies that these agrochemicals are not a “silver bullet” for combating this disease (Orton et al., 2011, Cools and Fraaije, 2008). Currently, STB control is achieved using the triazoles that target sterol 14α-demethylase enzymes of the ergosterol biosynthesis pathway, the succinate dehydrogenase
inhibitors (SDHIs), and the multisite targeting fungicide chlorothalonil (Cools and Fraaije, 2008, Orton et al., 2011, O’Driscoll et al., 2014, Torriani et al., 2015). However, strict European legislation on agrochemical application and registration of new active ingredients means that use of STB control fungicides may have to be restricted in the future. In addition fungicides such as SDHIs could be rendered ineffective due to potential target site mutations and furthermore the resulting fungicide resistant *Z. tritici* pathotypes could become hypervirulent (Scalliet et al., 2012). Therefore. It is crucial to manage fungicide resistance to minimise the threat posed by *Z. tritici* to global wheat production. In addition, future research efforts must concentrate on identifying new drug targets in *Z. tritici* for development of novel STB control chemistries.

**Figure 1.3** The *Z. tritici* genotype specific resistance in wheat.

The *Z. tritici* isolates IPO323 and IPO95052, which specifically infect bread and durum wheat, respectively, were inoculated on various wheat cultivars to screen for quantitative or qualitative STB disease resistance. Figure modified from Mirzadi Gohari et al., (2015) and Ponomarenko et al., (2011).

Fungicides are often used collectively with resistant wheat cultivars to diversify the modes of STB control. To date 21 wheat genes and 62 quantitative trait loci (QTL) have been identified as sources of qualitative and quantitative resistance, respectively, against specific *Z. tritici* genotypes (Figure 1.3) (Brown
et al., 2015). Thirteen wheat genes namely Stb1 to Stb12 and Stb15 confer specific strong genetic resistance against various Z. tritici isolates and are therefore attractive options for wheat breeding purposes (Orton et al., 2011, Brown et al., 2015, Brading et al., 2002). Such strong genetic resistance is often mediated through gene-for-gene interactions (Brown et al., 2015). These interactions impose an intense positive selection pressure on the pathogen, and often lead to rapid emergence of resistance pathotypes and breakdown of genetic resistance. Therefore, due to rapid evolution and genomic plasticity of Z. tritici, it is likely that the genetic resistance may be easily overcome if the individual Stb genes were to be used for breeding purposes (Orton et al., 2011, Brown et al., 2015). Indeed, some cases of breakdown of genetic resistance conferred by individual Stb genes have been reported in the USA (Cowger et al., 2000, Krenz et al., 2008) and the Netherlands (Kema and van Silfhout, 1997). Furthermore, such positive selection pressure is believed to have rendered all the known Stb genes ineffective against the European Z. tritici isolates (Arraiano et al., 2009, Brown et al., 2015).

In addition to the threat of breakdown of monogenic resistance, the association of strong STB resistance with lower yields (Torriani et al., 2015) has been a major hurdle in breeding new STB resistant wheat cultivars whilst maintaining and enhancing crop yields. These issues could be addressed by gene stacking or pyramiding, which involves insertion of multiple resistance genes into the same cultivar to increase the diversity of mode of actions and hence durability of genetic resistance (Mundt, 2014). This approach has already been applied to breed new wheat cultivars, which harbour multiple Stb genes and confer durable resistance against Z. tritici (Brown et al., 2015, Chartrain et al., 2004). Therefore, by stacking multiple Stb genes through novel
biotechnological approaches, it would be possible to extend the lifespan of current Stb genes whist also minimising the time required to breeding newer wheat cultivars.

Integrated disease control strategies comprising of fungicides applications, resistant wheat cultivars and agronomic practices are thought to be the best potential long-term solution for sustainable STB disease control (Torriani et al., 2015). Such strategies will be highly useful because new fungicides are not expected to arrive in the European market over next five years (Torriani et al., 2015). Agronomic practices such as modification of the crop canopy significantly reduce STB disease incidence by minimising inoculum dispersal (Arraiano et al., 2009). Such strategies can be implemented by growing tall wheat cultivars (Brown et al., 2015, Arraiano et al., 2009). However, consideration must be taken as tall wheat cultivars are prone to lodging, especially during heavy rains and winds, which causes yield losses; therefore yield penalties may remain unchanged although the cause of wheat yield loss may shift from STB disease to lodging (O'Driscoll et al., 2014). Mixtures of susceptible and resistant wheat cultivars also reduce losses to STB disease by 45% as compared to single cultivar cropping systems (Gigot et al., 2013). However, mixing the cultivars could affect consistency of quality traits such as gluten or protein content, which can be precisely maintained in single cultivar cropping systems. Therefore the practical difficulties in implementing such agronomic interventions present a major bottleneck in their wider uptake as a part of integrated STB control strategies (O'Driscoll et al., 2014).

1.7 Current status of functional genomics in Z. tritici
There has been a revolution in *Z. tritici* genomics during the last decade (Rudd, 2015). The whole genome sequence (Goodwin et al., 2011) has facilitated comparative genomics (Stukenbrock et al., 2011, Stukenbrock et al., 2012, Stukenbrock et al., 2007), which has provided new insights into the evolutionary history of *Z. tritici* (Rudd, 2015). Such comparative studies have opened new opportunities for identification of the positively selected genes, which encode proteins that play a critical role host specialization and virulence of *Z. tritici* (Poppe et al., 2015).

Although, significant advancements have been made in the identification potential virulence-associated SSPs (do Amaral et al., 2012, Mirzadi Gohari et al., 2015, Rudd et al., 2015), however, functional genetic analysis of these SSP-coding genes has been relatively restricted in *Z. tritici* (Rudd, 2015, Talbot, 2015). To date only 47 *Z. tritici* genes have been functionally characterised, many of which were only partially studied (Appendix 1). Out of these only 23 genes encode proteins that influenced virulence on wheat and majority of the proteins are conserved and known to play similar roles in other fungal pathogens (Rudd, 2015). The *Z. tritici* proteins that are known to influence virulence include conserved signalling MAPKs, enzymes in sterol biosynthesis pathway, one transcription factor, an ATP binding cassette (ABC) transporter and one secreted effector Mg3LysM. To date, only a single *Z. tritici* specific SSP encoded by the gene Zt80707 (Poppe et al., 2015) has been shown to reduce virulence of this pathogen. This illustrates existence of a major gap in the understanding of biology of *Z. tritici* infection. This gap stems from limited availability of the molecular tools for functional genomics in *Z. tritici* (Rudd, 2015, Talbot, 2015).
Availability of molecular tools for functional genomics research has been pivotal in revealing new insights into the biological mechanisms, which underlie virulence in major plant and human pathogens (Perez-Nadales et al., 2014, Dean et al., 2012, Giaever and Nislow, 2014). Availability of molecular tools and \textit{in vitro} phenotypic screening platforms facilitated in depth functional genomics analyses and consequently drove forward emergence of \textit{M. oryzae} as the model system for studying host-pathogen interactions (Wilson and Talbot, 2009). The \textit{M. oryzae} research community has hugely benefitted from the whole genome sequence that enabled for comparative genomics, proteomics and gene expression analyses. In addition, the protoplast based or \textit{Agrobacterium tumefaciens} mediated transformation (AMT), the \textit{Δku70} and \textit{Δku80} strains for high frequency gene targeting, ability to control gene expression by constructing temperature sensitive mutants and RNA interference for post transcriptional gene silencing have been key genetic manipulation tools that furthered functional analyses in \textit{M. oryzae}. In parallel, fluorescent reporters and antibodies for live cell imaging and subcellular immune-localisation facilitated elucidation of complex cytoskeletal remodelling event and their role in virulence of \textit{M. oryzae}. The \textit{in vitro} appressorium development assay has been a major tool for interrogation of infection related morphogenesis while the characterisation of \textit{M. oryzae} virulence benefitted from \textit{in planta} infection assays on rice, barley (\textit{Hordeum vulgare} L.) and the model grass species \textit{Brachypodium distachyon} (Wilson and Talbot, 2009). These tools enabled the \textit{M. oryzae} researchers to reveal invaluable insights into the virulence related role of cell-cycle checkpoints (Saunders et al., 2010), the role of cytoskeletal components in appressorium-mediated pathogenesis (Dagdas et al., 2012); and the effector secretion pathways (Giraldo et al., 2013).
The AMT of *Z. tritici* (Zwiers and De Waard, 2001, Motteram et al., 2009, Sidhu et al., 2015a) is currently the only method available for genetic modification of this fungus. AMT of *Z. tritici* is not a high throughput methodology such as the chemical transformation systems available for *Saccharomyces cerevisiae* (Gietz and Woods, 2002), *Neurospora crassa* (Park et al., 2011a) and *Candida glabrata* (Schwarzmuller et al., 2014). Low throughput and issues with reproducibility of the AMT protocol has been a major bottleneck in large-scale functional analyses of *Z. tritici*. As a result this has withheld the emergence of the Wheat- *Z. tritici* pathosystem as a model for studying the biology of the bi-phasic infection cycle.

Low gene targeting frequencies in *Z. tritici* has played the most important role in withholding the functional genomics through targeted gene deletions (Bowler et al., 2010). Highly efficient gene targeting was key to large-scale gene deletion studies in *S. cerevisiae* (Giaever et al., 2002), *N. crassa* (Colot et al., 2006) and *M. oryzae* (Kershaw and Talbot, 2009). The resulting knowledge from these fungal pathogens has revolutionised target based drug discovery and provided new insight into the eukaryotic biology. For example, systemic inactivation of genes encoding transcription factors (Colot et al., 2006) and the serine-threonine MAPK proteins (Park et al., 2011b) revealed novel gene regulatory and signalling mechanisms which underpinned morphogenesis, reproduction and stress resistance in *N. crassa*. In *A. fumigatus* efficient genomic modifications (da Silva Ferreira et al., 2006) revealed the role of cell wall α-(1,3)-glucans in PTI evasion (Beauvais et al., 2013), novel drug targets (Hu et al., 2007) and modes of action of drug molecules (Steinbach et al., 2007). Similarly, the ability to rapidly inactivate genes in *M. oryzae* was key to revealing the role of autophagocytosis in infection-associated morphogenesis.
Undoubtedly, the development of the *Z. tritici* strains which lack the non-homologous end joining (NHEJ) pathway of DNA repair and offer a high frequency of gene targeting (Bowler et al., 2010, Sidhu et al., 2015a) will open new opportunities for targeted genome modification and functional analysis. In fact, these *Z. tritici* NHEJ mutants are already proving instrumental in identification of essential genes and characterization of effectors (Sidhu et al., 2015a, Rudd et al., 2015, Marshall et al., 2011).

Until recently the precise transcriptional control of gene expression has been a major challenge in *Z. tritici* (Sidhu et al., 2015b, Marchegiani et al., 2015). The ability to regulate gene expression using inducible promoters enabled identification of essential genes and novel drug targets in *A. fumigatus* (Hu et al., 2007, Monteiro and De Lucas, 2010). Similarly inducible gene expression in *Candida albicans* was key to uncovering biological pathways that underpinned polarised growth (Chauvel et al., 2012) and regulators of infection-associated morphogenesis in response to hypoxia and temperature (Desai et al., 2015). Transcriptional control of gene expression using temperature-sensitive mutations, which enable growth at permissive temperatures, revealed that the mitotic checkpoint regulatory protein kinase *NIMA* and the protein encoded by major autophagy gene *ATG8* influence virulence of *M. oryzae* by affecting pre-penetration appresorium maturation (Veneault-Fourrey et al., 2006). Recently various constitutive and inducible promoters have been successfully deployed in *Z. tritici* to verify gene essentiality, functional analysis through gene overexpression and production of fusion proteins for cell biology and immunoprecipitation (Sidhu et al., 2015b, Marchegiani et al., 2015, Kilaru et al., 2015a, Cairns et al., 2015). This new capability to express/overexpress
genes of interest in a controlled manner will open new opportunities for functional analyses in *Z. tritici*.

The application of fluorescence cell biological techniques to study spatial and temporal interactions between *Z. tritici* and wheat has been virtually non-existent. Molecular tools and high-resolution live-cell biological imaging techniques will enable *Z. tritici* researchers to elucidate various enigmatic aspects such as role of putative SSPs in the Wheat- *Z. tritici* interactions (Rudd, 2015). For example, by tagging the *Ustilago maydis* effector See1 with a green fluorescent protein, Redkar et al., (2015) were able use cell biology to illustrate that the See1 interacts with the maize ortholog SGT1 to activate an immune response which leads to tumour formation and subsequently initiation of corn smut disease on maize seedlings (Redkar et al., 2015). Similarly, advanced cell biology revealed that *M. oryzae* engaged two distinct secretion pathways to deliver effectors for suppression of PTI in rice (Giraldo et al., 2013). By fluorescently tagging various proteins Giraldo et al., (2013) demonstrated that *M. oryzae* delivered apoplastic effectors through the endoplasmic reticulum to Golgi secretion pathway, while the delivery of cytoplasmic effectors was mediated through the biotrophy interfacial complex involving the exocyst components, Exo70 and Sec5 (Giraldo et al., 2013). Therefore, application of similar techniques to the Wheat- *Z. tritici* pathosystem could reveal the role played by *Z. tritici* effectors in modulation of host defences at various key stages of STB infection. Furthermore, cell biology was pivotal in revealing that the filamentous actin network and septin rings provided the rigid and curved membrane structure for appressorium mediated infection of rice by *M. oryzae* (Dagdas et al., 2012). Similarly, application of cell biological techniques could reveal the role of cytoskeletal remodelling during pre-penetration
morphogenesis, hyphal swelling and appressorium like structures, which are formed during infection by Z. tritici. The recently developed Z. tritici strains with fluorescently labelled cytoskeletal compartments (Kilaru et al., 2015c, Schuster et al., 2015a, Kilaru et al., 2015b, Schuster et al., 2015b, Guo et al., 2015) and vectors for protein tagging in Z. tritici (Sidhu et al., 2015b) will pave the way for application of cell biology to unravel the complex wheat- Z. tritici interactions.

1.8 Scope of the current study

The preceding literature review has identified several crucial aspects, which require in-depth investigation in order to develop a comprehensive biological understanding of the mode of infection adopted by Z. tritici. Current research was conducted to develop molecular tools for functional genomics in Z. tritici. In Chapter 3, I have reported construction and utilisation of the Z. tritici mutant strains, which facilitate rapid genome modification due to elevated frequency of homologous recombination (HR). In parallel I have described the utility of a new positive selection marker and a flexible cloning strategy for production of customised vectors for AMT of Z. tritici. The Chapter 4 describes the construction of a suite of 32 expression vectors and their application in functional genomics through gene overexpression and protein tagging for cell biology and immunoprecipitation techniques. In Chapter 5, I have reported the establishment of a strategy for controlled gene expression using inducible promoters and its deployment to reveal gene essentiality. Finally, the Chapter 6 describes a comprehensive characterisation of the glyoxylate cycle and illustrates the role of this pathway in pre-penetration spore to hyphal morphogenesis and virulence of Z. tritici. Overall, the tools developed in this study revealed that the fungus relies on lipolysis and the glyoxylate cycle to
produce energy and fuel the hyphal growth in order to initiate the STB disease in wheat. Thus, the current research has provided new molecular tools and furthered the biological understanding of the mechanisms that underpin the stealth pathogenesis of *Z. tritici*. 
Chapter 2

Materials and methods
All reagents were purchased from Sigma-Aldrich (Manchester, UK or Saint-Quentin, France) unless otherwise stated. Double distilled deionised water purified using Milli-Q® Water Purification System (Millipore UK Ltd., Watford, UK) was used as a solvent, unless otherwise stated. Growth media, solutions, and glass and plastic ware were autoclaved at 121 °C and 0.1 MPa or filter sterilized using 0.2 µm syringe filters. All cultures were incubated in Innova™ 42 or 44 rotary incubators (New Brunswick, St. Albans, UK).

2.1 Strains, databases and growth conditions

For long-term storage, microbial stocks were stored at – 80 °C in 50% glycerol (v/v in water). All bacterial and fungal strains constructed during this research project are maintained in the laboratory of Professor Ken Haynes at the University of Exeter.

2.1.1 Bioinformatics

The complete genome sequence of the Z. tritici isolate IPO323 (Goodwin et al., 2011) was accessed online using the genome portals maintained by the Joint Genome Initiative (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html) and the Ensembl fungi (http://fungi.ensembl.org/Zymoseptoria_tritici/Info/Index). Protein sequences were downloaded from the NCBI (National Centre for Biotechnology Information) repository (http://www.ncbi.nlm.nih.gov/genbank/) and aligned using the CLUSTAL Omega tool (http://www.ebi.ac.uk/Tools/msa/clustalo/). The MEGA6 suite (http://www.megasoftware.net/) was used for phylogenetic analysis.

2.1.1 Growth media recipes
The compositions of various growth media used in the current study are listed in Table 2.1. Growth media were prepared by dissolving the appropriate components in water to make final volume of one litre.

**Table 2.1 Growth media compositions**

<table>
<thead>
<tr>
<th>YPD broth or agar</th>
<th></th>
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<tbody>
<tr>
<td>Yeast extract</td>
<td>10 g L⁻¹</td>
</tr>
<tr>
<td>Bacteriological peptone</td>
<td>20 g L⁻¹</td>
</tr>
<tr>
<td>Glucose</td>
<td>20 g L⁻¹</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g L⁻¹ (For YPD agar only)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>YG broth or agar</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>10 g L⁻¹</td>
</tr>
<tr>
<td>Glucose</td>
<td>20 g L⁻¹</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g L⁻¹ (For YG agar only)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Synthetic Complete (SC) broth and agar</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast nitrogen base with ammonium sulfate and without amino acids (Formedium™, Hunstanton, UK)</td>
<td>6.9 g L⁻¹</td>
</tr>
<tr>
<td>Complete amino acid supplement (Formedium™, UK)</td>
<td>790 mg L⁻¹</td>
</tr>
<tr>
<td>Glucose</td>
<td>20 g L⁻¹</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g L⁻¹ (For SC agar only)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Minimal media (MM-Zt) broth and agar</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>20 g L⁻¹</td>
</tr>
<tr>
<td>Sodium nitrate</td>
<td>2 g L⁻¹</td>
</tr>
<tr>
<td>Potassium phosphate (dibasic)</td>
<td>1.5 g L⁻¹</td>
</tr>
<tr>
<td>Potassium phosphate (monobasic)</td>
<td>1 g L⁻¹</td>
</tr>
<tr>
<td>Magnesium sulfate heptahydrate</td>
<td>0.5 g L⁻¹</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.5 g L⁻¹</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>0.1 g L⁻¹</td>
</tr>
<tr>
<td>Thiamine (0.01% solution in water)</td>
<td>1 ml L⁻¹</td>
</tr>
<tr>
<td>Component</td>
<td>Concentration</td>
</tr>
<tr>
<td>--------------------------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Trace element solution</td>
<td>1 mL L(^{-1})</td>
</tr>
<tr>
<td>Vitamin solution</td>
<td>1 mL L(^{-1})</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g L(^{-1}) (For MM-Zt agar only)</td>
</tr>
<tr>
<td>Zinc sulfate heptahydrate</td>
<td>22 g L(^{-1})</td>
</tr>
<tr>
<td>Boric acid</td>
<td>11 g L(^{-1})</td>
</tr>
<tr>
<td>Manganese (II) chloride tetra hydrate</td>
<td>0.5 g L(^{-1})</td>
</tr>
<tr>
<td>Ferrous sulfate heptahydrate</td>
<td>0.5 g L(^{-1})</td>
</tr>
<tr>
<td>Cobalt (II) chloride hexahydrate</td>
<td>0.17 g L(^{-1})</td>
</tr>
<tr>
<td>Copper (II) sulfate pentahydrate</td>
<td>0.16 g L(^{-1})</td>
</tr>
<tr>
<td>Sodium molybdate dihydrate</td>
<td>0.15 g L(^{-1})</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid</td>
<td>5 g L(^{-1})</td>
</tr>
<tr>
<td>Vitamin solution (pH = 6.0)</td>
<td></td>
</tr>
<tr>
<td>Biotin</td>
<td>0.01 g L(^{-1})</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>0.01 g L(^{-1})</td>
</tr>
<tr>
<td>Thiamine</td>
<td>0.01 g L(^{-1})</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.01 g L(^{-1})</td>
</tr>
<tr>
<td>p-aminobenzoic acid</td>
<td>0.01 g L(^{-1})</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.01 g L(^{-1})</td>
</tr>
</tbody>
</table>

MM-Zt+NH\(_4\), MM-Zt+Glu or MM-Zt+Gln media were prepared by replacing sodium nitrate in MM-Zt media with 23 mM ammonium nitrate, glutamate or glutamine respectively.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal medium (BM) broth or agar (Yang and Naqvi, 2014)</td>
<td></td>
</tr>
<tr>
<td>(pH to 6.0 with 1 M phosphate buffer)</td>
<td></td>
</tr>
<tr>
<td>Yeast nitrogen base without amino acids</td>
<td>1.7 g L(^{-1})</td>
</tr>
<tr>
<td>or ammonium sulfate (Formedium™, UK)</td>
<td></td>
</tr>
<tr>
<td>Asparagine</td>
<td>2 g L(^{-1})</td>
</tr>
<tr>
<td>Ammonium nitrate</td>
<td>1 g L(^{-1})</td>
</tr>
<tr>
<td>Glucose</td>
<td>10 g L(^{-1})</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g L(^{-1}) (For BM agar only)</td>
</tr>
</tbody>
</table>

Induction media (IM) broth or agar

(pH = 5.6)
Potassium phosphate (monobasic) 10 mM
Potassium phosphate (dibasic) 10 mM
Sodium chloride 2.5 mM
Magnesium sulfate heptahydrate 2 mM
Calcium chloride dihydrate 0.7 mM
Ferrous sulfate heptahydrate 10 µM
Ammonium sulfate 4 mM
Glucose 10 mM
2-(N-Morpholino)-ethanesulfonilic acid 40 mM
Glycerol 0.5 % (v/v)
Agar 20 g L\(^{-1}\) (For IM agar only)

<table>
<thead>
<tr>
<th>Synthetic Complete media without uracil (SC-URA) broth or agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast nitrogen base with ammonium sulfate and without amino acids (Formedium™, Hunstanton, UK) 6.9 g L(^{-1})</td>
</tr>
<tr>
<td>Complete amino acid supplement without uracil (Formedium™, UK) 790 mg L(^{-1})</td>
</tr>
<tr>
<td>Glucose 20 g L(^{-1})</td>
</tr>
<tr>
<td>Agar 20 g L(^{-1}) (For SC agar only)</td>
</tr>
</tbody>
</table>

| Potato dextrose (PD) broth and agar were purchased from Sigma-Aldrich, UK |
| Czapek Dox (CD) broth and agar were purchased from Sigma-Aldrich, UK |
| Lysogeny broth (LB) and agar were purchased from Formedium™, Hunstanton, UK. |

2.1.2 Growth conditions

The *Z. tritici* isolate IPO323 (Kema and van Silfhout, 1997) was used as the wild type (WT). To produce yeast-like budding spores of *Z. tritici*, a loop full of fungal glycerol stock was inoculated into broth or agar media and cultures were incubated for 6 days at 15 or 18 °C; liquid cultured were shaken at 180 rpm. To harvest the spores, liquid cultures were centrifuged at 13000 rpm for 5
minutes at 4 °C. Spores were harvested from agar plates by adding 1 mL sterile water to agar surface and gently scraping the fungal growth into a suspension using a sterile cell spreader. Spore suspension was filtered through sterile miracloth (pore size = 22 – 25 µm) (Merck Millipore, Nottingham, UK) and centrifuged at 13000 rpm for 5 minutes at 4 °C to pellet the spores. Fungal mycelia were produced by inoculating *Z. tritici* into MM-Zt or CD broth followed by 6 day incubation at 25 °C and 100 rpm. Mycelia were harvested by filtering the culture through sterile miracloth.

*Escherichia coli* strain DH5α (*fhuA2 lacΔU169 phoA glnV44 Φ80* *lacZΔM15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*) (Hanahan, 1985) and *ccdB* resistant (*ccdB*<sup>R</sup>) strain TOP10 (*FcroA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str<sup>R</sup>) endA1 λ<sup>+</sup>*) (Life technologies Ltd., Paisley, UK) were used for plasmid propagation. *E. coli* cultures were grown at 37 °C (180 rpm for liquid cultures) in LB broth or agar supplemented with appropriate antibiotics (100 µg mL<sup>−1</sup> of either kanamycin, ampicillin or gentamycin).

*Agrobacterium tumefaciens* strain EHA105 was cultured at 30 °C (180 rpm for liquid cultures) in LB medium or IM broth or agar (Zwiers and de Waard, 2001). *A. tumefaciens* cultures were amended wither with 100 µg mL<sup>−1</sup> rifampicin (stock 100 mg mL<sup>−1</sup> dissolved in DMSO) and/or 100 µg mL<sup>−1</sup> kanamycin.

*Saccharomyces cerevisiae* strain BY4741 (*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*) (Brachmann et al., 1998) was cultured at 30 °C and 180 rpm for liquid cultures. *S. cerevisiae* cultures were grown in YPD or SC-URA.
2.2 Nucleic acid manipulations

2.2.1 Z. tritici genomic DNA extraction

Genomic DNA was extracted from spores grown on YPD or YG agar media for 6 days at 15 °C. Spores were harvested into a suspension and counted using a haemocytometer. Appropriate volume containing 5x10^8 spores was added into a sterile 2 mL Eppendorf Safe Lock Tube™ (Fisher Scientific, Loughborough, UK). Tubes were centrifuged at 4 °C and 13000 rpm for 5 minutes and supernatant was removed without disturbing the spore pellet. Pellet was suspended in 400 µL of Z. tritici lysis solution which contained 2 % (v/v) TRITON X, 1 % (v/v) sodium dodecylsulfate, 100 mM sodium chloride, 10 mM Tris-HCl (pH 8.0), 2 mM EDTA and 50 µg/mL RNase A in water. Then, 200 µg glass beads (acid washed, 425-600 µm) were added and tubes were vigorously shaken for 2 minutes using the FastPrep™24 sample preparation system (MP Biomedical, Manchester, UK). Subsequently, 400 µL phenol:chloroform: Isoamyl alcohol [(25:24:1)(v/v)] was added and tubes were inverted to mix the contents and centrifuged for 15 minutes at 13000 rpm and 4 °C. A 500 µL aliquot of top aqueous phase was transferred into a sterile 2 mL tube containing 500 µl chloroform and tubes were inverted to mix contents and maintained on ice for 5 minutes. Tubes were centrifuged for 10 minutes and 350 µL of top aqueous phase was transferred into another sterile 2 mL tube. To precipitate the DNA, 35 µL 3 M sodium acetate (pH 5.2) and 1200 µL ice cold ethanol (100%) were added and tubes were stored at – 20 °C for 12 hours. After this, tubes were centrifuged at 4 °C and 13000 rpm for 15 minutes to pellet the DNA and supernatant was discarded. To wash the DNA pellet, 1 mL ice cold ethanol (70 % (v/v) in water) was added and tubes were centrifuged at
13000 rpm and 4 °C for 5 minutes. Supernatant was carefully removed by pipetting and tubes were incubated at room temperature for 10 minutes to evaporate residual ethanol. DNA pellet was suspended in 100 µL nuclease free TE (pH 8.0) (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and samples were stored at – 20 °C until subsequent analyses.

**2.2.2 RNA extraction and cDNA synthesis**

Double autoclaved glass and plastic ware was used for RNA extraction. All solutions were made using double autoclaved diethyl pyrocarbonate (DEPC) treated deionised water.

Total RNA was extracted from freeze dried Z. tritici spores or mycelia. Spores were harvested from YPD or YG agar plates as previously stated. Mycelia were grown for 6 day old MM-Zt or CD cultures grown at 25 °C and 100 rpm and harvested by filtering the cultures through sterile miracloth. Fungal biomass was mechanically disrupted in liquid nitrogen using a metal ball mill and/or cell disruptor. Total RNA was isolated using RNeasy® Plant Mini Kit (Qaigen, UK) or using TRIzol® Reagent (Life Technologies, UK) following manufacturer’s instructions. Total RNA was suspended in DEPC water and stored in - 80 °C.

Total RNA was treated with RNase-Free DNase (Qaigen, Manchester UK) following manufacturer’s instructions. To test complete degradation of genomic DNA, real-time PCR analysis was carried out using cDNA specific primers. RNA integrity was checked on agarose gel and was quantified using the Nanodrop™ 2000 spectrophotometer (Thermo Scientific, London, UK).
Double stranded cDNA was synthesized from 1 µg of total RNA using random hexamers or gene specific primers with AffinityScript Q-PCR cDNA Synthesis Kit (Agilent technologies, Wokingham, UK) following the manufacturer’s protocol.

2.2.3 Restriction digestion using endonucleases

All restriction endonucleases were purchased from New England Biolabs (NEB), Hitchin, UK. To digest plasmid DNA, reactions containing 5 µg of DNA were assembled as per manufacturer’s instructions and incubated at 37 °C for 3 hours. Restriction endonucleases compatible with heat inactivation were denatured by incubation at 65 - 80 °C for 10 minutes. For Southern blot analysis, 20 µg fungal genomic DNA was digested for 12 hours at 37 °C.

2.2.4 Agarose gel electrophoresis

DNA was size fractioned on agarose gels (0.8 % - 2 % (w/v) agarose in TAE buffer which contained 40 mM Tris, 20 mM acetic acid and 1 mM EDTA dissolved in water). DNA was separated on 2% agarose gels and while 0.8% gels were used for southern blot analysis and gel purification of DNA. To stain the DNA gels, 1 µL ethidium bromide was added to 50 mL molten agarose before casting each gel. DNA samples were mixed with 6x Blue Gel Loading Dye (NEB, UK) prior to loading on gel. The Quick-load™ 1 Kb DNA ladder or Quick-load™ 100 bp DNA ladder (NEB, UK) was used as DNA size standard. Gel electrophoresis was carried out in TAE buffer at 100 volts using PowerPac™ Universal Power Supply (Bio-Rad Laboratories Ltd. Hemel Hempstead, UK). Agarose gels were visualised in presence of ultraviolet (UV) radiation and images were acquired using GBOX Chemi XX6 imaging system (Syngene, Cambridge, UK).
2.2.4 Polymerase chain reaction

Polymerase chain reaction (PCR) was used to amplify DNA fragments for genetic cloning and to confirm the genetic modification events in the *Z. tritici* genome. DNA fragments used cloning were amplified from genomic DNA or cDNA using the Phusion® high fidelity polymerase (NEB, UK) as per manufacturer’s instructions. The GoTaq® flexi DNA polymerase (Promega, Southampton, UK) was used for colony PCR experiments. All PCR reactions were conducted in Techne TC5000 PCR system (Bibby Scientific, Staffordshire, UK). All PCR primers used in this study were purchased from Sigma-Aldrich and are shown in Appendix 2. Typical PCR reactions contained 50 – 100 ng template, 1 µM primer mixture. 10 nM magnesium chloride, 2 mM dNTPs, 1-2 units of DNA polymerase and PCR grade water (Sigma-Aldrich) was added to make final volume of 50 µL. PCR reactions using the GoTaq® flexi DNA polymerase were denatured for 5 minutes at 95 °C followed by 35 cycles with each cycle involving 30 second denaturation at 95 °C, 30 second primer annealing at 60 – 65 °C and extension at 1 minute/kb at 72 °C; followed by a final 10 minute extension at 72 °C and finial hold at 4 °C. Reactions involving Phusion® high fidelity polymerase were denatured at 98 °C for 30 seconds followed by 35 cycles with each cycle involving denaturation 98 °C for 10 seconds, primer annealing at 65 – 72 °C for 30 seconds and extension at 72 °C for 30 seconds/kb, and a final 10 minute extension at 72 °C followed by final hold at 4 °C.

2.2.5 Extraction of DNA from agarose gels

DNA was separated by gel electrophoresis using 0.8 % agarose gel and DNA fragments of interest were recovered from using silicon dioxide and
sodium iodide method described by Li et al., (2010) (Li et al., 2010). Agarose gel slice containing DNA fragment of interest was excised and transferred into sterile 1.5 mL microcentrifuge tube and weighed. For each 100 mg agarose gel, 300 µL 6 M sodium iodide solution was added and tubes were incubated at 70 °C for 5 minutes to dissolve agarose. Subsequently, 10 µL of silicon dioxide mixture (100 mg mL⁻¹ (w/v) silicon dioxide in sterile water) was added and contents were mixed by inversion and incubated at room temperature for 2 minutes. Tubes were centrifuged at 13000 rpm for 10 seconds and supernatant was removed without disturbing the pellet. Pellet was washed by adding 500 µL of wash solution (50% ethanol (v/v), 10 mM Tris-HCl (pH 7.5), 100 mM NaCl and 1 mM EDTA dissolved in water) and tubes were vortexed to suspend the pellet. Tubes were centrifuged at 13000 rpm for 10 seconds to pellet silicon dioxide matrix. Supernatant was removed by pipetting and tubes were dried at 70 °C for 5 minutes to evaporate residual liquid. Subsequently, the silicon dioxide pellet was suspended in 40 µL TE (pH 8.0) and tubes were maintained on 70 °C for 2 minutes followed by centrifugation at 13000 rpm for 2 minutes. Supernatant containing eluted DNA was carefully transferred into a sterile 1.5 mL microcentrifuge and recovered DNA was analysed by agarose gel electrophoresis.

2.3 Genetic cloning

2.3.1 Blunt and sticky end ligation

Blunt ended PCR amplified products were directly cloned into the sequencing vector pCR®-Blunt II-TOPO® vector (Life technologies, UK) using the Zero Blunt TOPO® PCR cloning Kit (Life technologies, UK) by following manufacturer’s instructions. The T4 DNA ligase (NEB, UK) was used for
standard ligation of restriction digested products containing 5’ or 3’ overhangs into appropriately digested destination vectors. All ligation reactions were assembled as per manufacturer’s instruction and incubated for 12 hours 16 °C followed by heat inactivation at 65 °C prior to transformation into appropriate *E. coli* strain.

### 2.3.2 Yeast recombinational cloning using *S. cerevisiae*

A modified version of yeast recombinational cloning protocol established by Collopy et al., (2010) (Collopy et al., 2010) was used to recombine multiple DNA fragments in *S. cerevisiae*.

DNA fragments used for recombination were amplified by PCR from the template DNA using primers, which contained 30 nucleotides complementary to the following and proceeding fragments that were to be recombined. The gel purified PCR products (500 ng each) and restriction digested vector (100 ng) were mixed in a 1.5 mL microcentrifuge tube to a final volume of 15 µL and co-transformed in to *S. cerevisiae*.

### 2.3.2.1 Transformation of *S. cerevisiae*

The *S. cerevisiae* strain BY4741 was inoculated from glycerol stock into 3 mL YPD broth and grown for 12 hours at 30 °C and 180 rpm. A 2 mL aliquot of overnight culture was inoculated into a 48 mL fresh YPD broth in a sterile 250 mL Erlenmeyer flask and cultured at 30 °C and 180 rpm for 4 hours. Culture were transferred into 50 mL falcon tubes and centrifuged at 4000 rpm for 2 minutes to pellet the *S. cerevisiae* cells. Cell pellet was suspended in 50 mL sterile water and centrifuged at 4000 rpm for 2 minutes. Supernatant was removed and cell pellet was suspended 2 mL sterile water.
S. cerevisiae was transformed using lithium acetate and polyethylene glycol (PEG) method as described by Gietz and Woods (2002). Transformation reactions were assembled in 1.5 mL microcentrifuge tubes containing transforming DNA fragments into which following were added; 50 µL of denatured single stranded salmon sperm DNA (stock solution 2 mg mL⁻¹ (w/v) in TE (pH 8.0)), 50 µL S. cerevisiae cells suspension, 36 µL of sterile 1 M lithium acetate solution and 250 µL of sterile PEG 3000 (50 % (w/v) in water). Contents were mixed gently by pipetting and tubes were incubated at 30 °C for 30 minutes followed by a heat shock at 45 °C for 15 minutes. Afterwards, tubes were centrifuged at 4000 rpm for 5 minutes to pellet the S. cerevisiae cells and supernatant was removed by pipetting. Cell pellet was suspended in 200 µL sterile water and plated on SC-URA agar and plates were incubated for 48 – 72 hours at 30 °C.

2.3.2.2 Isolation of plasmid DNA from S. cerevisiae

The recombinant plasmids were extracted from the uracil prototrophic S. cerevisiae transformants using the lyticase mediated enzymatic digestion and QAIprep spin miniprep kit (Qaigen, UK) protocol described by Singh and Weil (2002). A single S. cerevisiae colony was inoculated into 5 mL SC-URA broth and grown for 12 hours at 30 °C and 180 rpm. Cultures were centrifuged at 13000 rpm for 5 minutes; supernatant drained and cell pellet was resuspended in 200 µL resuspension solution (P1 buffer in QAIprep spin miniprep kit (Qaigen, UK)). Suspension was transferred into a sterile 1.5 mL microcentrifuge tube into which 100 µL of lyticase solution (Arthrobacter luteus lyticase powder 5 mg mL⁻¹ (w/v) in 1.2 M sorbitol solution, 0.1 M sodium phosphate buffer (pH 7.4)) was added, followed by incubation at 37 °C for 30 minutes. Next, 300 µL of lysis solution (P2 buffer QAIprep spin miniprep kit) was added and contents
were mixed by inversion and incubated at room temperature for 10 minutes. To neutralise the lysate, 420 µL of neutralization solution (N3 buffer in QIAprep spin miniprep kit) was added and contents were mixed by inversion followed by centrifugation at 13000 rpm for 10 minutes. Subsequently, 700 µL supernatant was applied to the QIAprep plasmid mini column and centrifuged at 13000 rpm for 1 minute and flow through was discarded. A 500 µL aliquot of wash buffer (PE buffer in QIAprep spin miniprep kit) added and column was centrifuged at 13000 rpm for 1 minute. After discarding the flow-through, columns were dried by centrifuging at 13000 rpm for 1 minute. Finally, the plasmid DNA was eluted by adding 30 µL TE (pH 8.0) to the QIAprep plasmid mini column.

2.3.3 Gateway® recombination cloning

The Gateway® recombination cloning was carried out using the BP Clonase™ or LR Clonase™ enzymes mixtures (Life Technologies, UK) following manufacturer’s instructions.

For Gateway® BP recombination cloning, open reading frames (ORFs) were PCR amplified from the template DNA/cDNA using forward and reverse primers that contained the attB1 and attB2 sites followed 20 bp complementary to 5’ and 3’ of the ORF, respectively. PCR amplification of the target ORF was checked by agarose gel electrophoresis and PCR product was precipitated using ethanol and resuspended in 20 µL TE (pH 8.0). BP recombination reactions were set up by adding 5 µL PCR product, 1 µL donor vector pDONR207 (Life Technologies, UK), 2 µL BP Clonase™ enzyme mix and 2 µL TE (pH 8.0) into a sterile PCR tube. Reaction mixtures were incubated for 12 hours at 25 °C and subsequently transformed into E. coli strain DH5α. Transformation mixtures were plated on LB agar containing 100 µg mL⁻¹ gentamycin to select transformants harbouring recombinant entry clones.
To construct the expression clones by the Gateway® LR recombination cloning, each reaction was assembled by adding 5 µL (100 ng µL⁻¹) Sanger sequence confirmed entry clones, 1 µL (100 ng µL⁻¹) Gateway® compatible expression vector, 2 µL LR Clonase™ enzyme mix and 2 µL TE (pH 8.0) into a PCR tube. Reaction mixture was incubated for 24 hours at 25 °C followed by transformation into *E. coli* strain *DH5α*. Mixture was plated on LB agar media containing either 100 µg mL⁻¹ kanamycin or ampicillin to select transformants harbouring recombinant expression clones.

### 2.3.4 Transformation of *E. coli*

The glycerol stock of appropriate *E. coli* strain was inoculated into 5 mL LB broth and cultured overnight at 37 °C and 180 rpm. A 1 mL aliquot of this overnight culture was inoculated into 100 mL fresh LB broth in a sterile 500 mL Erlenmeyer flask and grown for 5 hours or till culture reached OD₅₅₀ 0.48. Culture was chilled at 0 – 2 °C for 10 minutes and transferred into 50 mL falcon tubes and centrifuged at 4000 rpm and 4 °C for 5 minutes. Supernatant was discarded and the *E. coli* cell pellet was suspended in 10 mL of ice-cold 100 mM calcium chloride dihydrate solution and incubated at 0 – 2 °C for 20 minutes. Suspension was centrifuged at 4000 rpm and 4 °C for 5 minutes and supernatant was discarded. Subsequently, cell pellet was suspended in 5 mL ice-cold solution of 100 mM calcium chloride dihydrate. Competent cell suspension was divided into 500 µL aliquots in microcentrifuge tubes and stored at – 80 °C until further use.

The *E. coli* competent cell suspension was defrosted on ice and 50 µL aliquot was added to a microcentrifuge tube containing either 5 µL of plasmid isolated from *S. cerevisiae* or 1 µl (100 ng µL⁻¹) of plasmid DNA or 10 µL of T4 ligation reaction mixture or 5 µL of Gateway® reaction mixture. Transformation
mixture was incubated on ice for 30 minutes followed by 42 °C heat shock for
30 seconds and a subsequent 5 minute incubation on ice. A 200 µL aliquot of
sterile LB broth was added and transformed cells were regenerated for 2 hours
at 37 °C and 180 rpm. Transformation mixture was plated on LB agar
containing appropriate antibiotic and plates were incubated at 37 °C for 12 – 16
hour.

2.3.5 Extraction of plasmid DNA from *E. coli*

All plasmid were isolated from *E. coli* using QAIprep spin miniprep kit
(Qaigen, UK) as per manufacturer’s instructions. Briefly, a single *E. coli* colony
or a loop of glycerol stock was inoculated into 5 mL LB broth amended with
appropriate antibiotic and cultured overnight at 37 °C and 180 rpm. Culture was
centrifuged 4000 rpm and supernatant discarded. Cell pellet was resuspended
in 250 µL resuspension solution (P1 buffer). Next, 300 µL lysis solution (P2
buffer) was added and contents were mixed by inverting followed by addition of
350 µL neutralization solution (N3 buffer). Mixture was centrifuged at 13000 rpm
for 10 minutes and 750 µL of the supernatant was applied to the QAIprep
plasmid mini column and centrifuged at 13000 rpm for 1 minute. Flow through
was discarded and column was washed by adding 500 µL wash buffer (PE
buffer) and centrifuged at 13000 rpm for 1 minute. After discarding the flow
through, column was dried by centrifuging at 13000 rpm for 1 minute. Column
was transferred into a sterile 1.5 mL microcentrifuge tube and 30 µL TE (pH
8.0) added followed by centrifugation at 13000 rpm for 1 minute. The
concentration and purity of eluted plasmid DNA was analysed by Nanodrop
spectrophotometer and/or gel electrophoresis. Subsequently, 200 ng plasmid
DNA was used for nucleotide sequence analysis using the Sanger sequencing
service (Eurofins Genomics, Ebensburg, Germany).
2.4 Agrobacterium mediated transformation of *Z. tritici*

Agrobacterium mediated transformation (AMT) of *Z. tritici* was carried out with modification to the AMT protocols described by Bowler et al., (2010), Motteram et al., (2009) and Zwiers and De Waard (2001).

2.4.1 Preparation of *A. tumefaciens* competent cells

*A. tumefaciens* glycerol stock was inoculated into 5 mL LB broth containing 100 µg mL$^{-1}$ of rifampicin and grown for 12 hours at 30 °C and 250 rpm. A 2 mL aliquot of overnight cultures was inoculated into 48 mL of LB broth amended with 100 µg mL$^{-1}$ rifampicin in a sterile 250 mL Erlenmeyer flask and grown at 30 °C and 250 rpm for 6 – 8 hours until culture reached OD$_{600}$ 0.6. Culture was chilled on ice for 10 minutes and transferred into a chilled sterile 50 mL falcon tubes. Culture was centrifuged for 5 minutes at 4 °C and 4000 rpm and supernatant discarded carefully without disturbing the *A. tumefaciens* cell pellet. Subsequently, cell pellet was suspended in 1 mL 20 mM calcium chloride solution and 100 µL aliquots of the *A. tumefaciens* competent cell suspension were stored at – 80 °C until further use.

2.4.2 Transformation of *A. tumefaciens*

*A. tumefaciens* competent cell suspension were defrosted on ice and 50 µL aliquot was mixed with 10 µL of the plasmid DNA in a sterile 1.5 mL microcentrifuge tube and incubated on ice for 10 minutes. Transformation mixture was snap frozen in liquid nitrogen for 1 minute followed by incubation at 37 °C for 5 minutes. Subsequently, 500 µL of fresh LB broth was added and mixture was incubated at 30 °C and 180 rpm for 2 hours. Finally, 200 µL aliquot of transformation mixture was spread on LB agar containing 100 µg mL$^{-1}$
kanamycin and rifampicin. Plates were incubated at 30 °C and transformants emerged after 48 – 72 hours. A single transformant colony was inoculated into 5 mL LB broth containing 100 µg mL\(^{-1}\) kanamycin and rifampicin and grown for 24 hours at 30 °C and 180 rpm. A 1 mL aliquot of this overnight culture was mixed with 1 mL sterile 100% glycerol to make glycerol stock which was stored at – 80 °C.

For high-throughput of *A. tumefaciens* transformation in 96 well plates, 5 µL plasmid DNA was mixed with 30 µL competent cells in each well and plate was incubated on ice for 10 minutes. Plate was snap frozen in liquid nitrogen for 1 minute followed by 5 minute incubation at 37 °C. Using a multichannel pipette, 165 µL LB broth was added into each well and plate was incubated for 2 hours at 30 °C and 180 rpm. Subsequently, 50 µL transformation mixture from each well was spread on individual LB agar plates containing 100 µg mL\(^{-1}\) kanamycin and rifampicin.

### 2.4.3 AMT of *Z. tritici*

The appropriate *A. tumefaciens* strain harbouring the desired plasmid was streaked on LB agar containing 100 µg mL\(^{-1}\) kanamycin and rifampicin and plates were incubated at 30 °C for 48 hours. A loop full the *A. tumefaciens* growth was inoculated into 10 mL LB broth containing 100 µg mL\(^{-1}\) kanamycin and rifampicin and grown for 12 to 16 hours at 30 °C and 180 rpm. Cultures with OD\(_{660}\) between 0.95 – 1.0 were diluted to OD\(_{660}\) 0.10 by IM broth amended with 100 µg mL\(^{-1}\) kanamycin and rifampicin, and 40 µg mL\(^{-1}\) acetosyringone (3′, 5′-Dimethoxy-4′-hydroxyacetophenone) (stock 40 mg mL\(^{-1}\) (w/v) in DMSO). Diluted *A. tumefaciens* cultures were grown at 30 °C and 180 rpm for 3-5 hours or until cultures reached an OD\(_{660}\) 0.25 – 0.35.
*Z. tritici* spores were harvested from YPD or YG agar plates as previously described. Spore suspension was centrifuged and supernatant was discarded. Spore pellet was suspended in IM broth and after counting the spores, the concentration was adjusted to $1 \times 10^7$ spores mL$^{-1}$ in IM broth containing 100 µg mL$^{-1}$ kanamycin and rifampicin, and 40 µg mL$^{-1}$ acetosyringone.

A 300 µL of the *A. tumefaciens* cultures at OD$_{660}$ 0.25 – 0.35 was mixed with 100 µL ($10^6$ spores) of *Z. tritici* spore suspension and mixture was spread on top of the sterile cellophane discs placed on IM agar plates containing 100 µg mL$^{-1}$ kanamycin and 40 µg mL$^{-1}$ acetosyringone. Plates were incubated at 25 °C for 48 hours. Subsequently, cellophane discs were transferred to MM-Zt, CD or BM agar amended with appropriate antifungal selection agent and following antibiotics; 100 µg mL$^{-1}$ of ampicillin, cefotaxime, streptomycin and timentin. After transferring the discs, selection plates were incubated at 15 to 18 °C until the putative *Z. tritici* transformants were observed (typically up to 10 - 12 days). Putative transformants were sub-cultured for two further rounds on selective media to eliminate untransformed background growth.

### 2.5 Southern blot analysis

Southern blot analysis (Southern, 1975) was carried out to confirm the integration of the transforming DNA into the *Z. tritici* genome. Digoxigenin (DIG) dUTP labelled DNA probes used for Southern blot analysis were synthesized by PCR using PCR DIG Probe Synthesis Kit (Roche Diagnostics Ltd., Burgess Hill, UK) following the manufacturer’s instructions. To eliminate amplification of the nonspecific probes, the primers used for probe synthesis were designed using NCBI primer blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) with default
settings. All DIG labelled probes were gel purified to eliminate template, primers and DIG-dUTPs.

Fungal genomic DNA was digested with appropriate restriction endonucleases and size fractioned via gel electrophoresis on 0.8% agarose gel (without ethidium bromide) at 80 volts for 4 to 8 hours. Subsequently, gel was stained by immersing in TAE containing 10 µL of ethidium bromide (stock 1 µg mL⁻¹ (w/v) in water) for 20 minutes and imaged in presence of UV radiation.

Size fractioned DNA was depurinated by immersing the gel in 0.25 M HCl for 15 minutes followed by 30-minute incubation in denaturation solution (0.4 M sodium hydroxide and 0.6 M sodium chloride) at room temperature. Gel was incubated in neutralization buffer (1.5 M sodium chloride, 0.5 M Tris-HCl (pH 7.5)) for 30 minutes and equilibrated for 10 minutes in 20 X SSC (3M sodium chloride and 0.3 M sodium citrate) at room temperature. Subsequently, DNA was transferred from gel to Hybond-N+ positively charged nylon membrane (GE Healthcare Life Sciences, Chalfont, UK) by capillary action, using 20 X SSC, for 12 – 16 hours. Finally, DNA was cross-linked by exposing the membrane to UV radiation using the Bio-Link BLX 254 UV Crosslinker (VWR International Limited, Lutterworth, UK).

After crosslinking, membrane was incubated in hybridization buffer (Roche, UK) for 30 minutes at 65 °C followed by overnight incubation at 65 °C in probe hybridisation buffer (hybridization buffer containing denatured DIG-dUTP labelled probe). Next, membrane was washed in low stringency buffer (2x SSC (made by diluting 20x SSC to 1:10 (v/v) in water) and 0.1 % (v/v) SDS) for 10 minutes at room temperature. Membrane was then incubated in high stringency buffer (0.5 x SSC (made by diluting 20x SSC to 1:40 (v/v) in water)
and 0.1 % (v/v) SDS) at 65 °C for 30 minutes followed by a 2 minute incubation in the wash buffer (0.3% (v/v) Tween-20, 0.1 M maleic acid and 0.15 M sodium chloride; pH 7.5). Membrane was incubated in the blocking solution (1% (w/v) Roche blocking reagent (Roche, UK), 0.1 M maleic acid and 0.15 M sodium chloride; pH 7.5) for 1 hour at room temperature followed by incubation in antibody solution (0.001 % (v/v) polyclonal anti-Digoxigenin antibody in the blocking solution) for 30 minutes. Next, membrane was washed for 30 minutes in the wash buffer and subsequently equilibrated in the detection buffer (0.1 M Tris-HCl and 0.1 M sodium chloride; pH 9.5) for 3 to 5 minutes at room temperature. Finally a 1 mL chemiluminescent substrate (Roche, UK) was spread on the membrane followed by a 5-minute incubation at room temperature. Membrane was exposed to UV radiation and imaged using G:BOX Chemi XX6 imaging system.

2.6 Virulence assay

The virulence assay was conducted on the wheat seedlings as previously described by Motteram et al., (2009) and Keon et al., (2007). The seeds of bread wheat cultivar Avalon or Riband were sown in John Innes No.2 compost medium. Seedlings were grown at 16 °C, 75 – 90 % relative humidity and 16:8 hour day: night cycles for 17 days in the MLR-352 environmental testing chamber (Panasonic Biomedical, Leicestershire, UK).

Z. tritici spores used for virulence assays were harvested from YPD or YG agar plates incubated at 15 °C for 6 days. Spore suspension was enumerated and concentration was adjusted to $10^7$ spores mL$^{-1}$ in sterile inoculation solution (0.1% (v/v) Tween-20 in water). Spore suspension was evenly inoculated on the adaxial side of the second true leaf of the wheat
seedlings using a sterile paintbrush or a cotton bud. The inoculated plants were maintained for 96 hours at 16 °C and 100% relative humidity followed by reduction in relative humidity to 75% for up to 21 days. Progress of infection was visually inspected at 24-hour intervals from 12 days after inoculation (DAI). At 21 DAI leaves were excised and fixed to transparent plastic sheets and maintained in 100% relative humidity for 24 hours to initiate the release of the pycnidiospore containing cirrhi. Images were acquired using the Leica M205FA stereomicroscope (Leica, Milton Keynes, UK) and/or the Epson scanner (Epson, Hertfordshire, UK). Disease symptoms were analysed visually and by manually counting the pycnidial lesions on the leaf surface.

2.7 Phenotypic screen

All in vitro phenotypic screens were carried out using Z. tritici spores harvested from YPD, YG or SC agar following 6-day growth at 15 °C. Serially diluted spore suspensions were spotted on agar media amended with appropriate stress conditions. Plates were incubated at 15 to 18 °C for 5 to 8 days and Images were acquired in presence of white light using the G-BOX Chemi XX6 imaging system. To monitor in vitro spore germination and hyphal formation, serially diluted spore suspensions were spotted on agarose bed on glass slides and maintained in a humidity chamber (>85 % relative humidity) at 15-25 °C.

2.8 Microscopic analysis

2.8.1 Epifluorescence microscopy

Epifluorescence microscopy and quantitative analysis of fluorescence was conducted as described by Bielska et al., (2014) and Schuester et al., (2011).
Z. tritici spores were harvested from 72 hour-old YPD, YG or SC cultures grown at 15 to 18 °C and 180 rpm. Spores were spotted on agarose cushion (2% (w/v) agarose in water) on a glass slide. Slides were exposed to a 488-nm/70 mW solid-state laser at 20% laser intensity to excite the EGFP fluorescence. Micrographs were acquired by applying the appropriate fluorescence filter at 200 ms exposure using the Olympus IX81 motorized inverted spinning disc microscope (Olympus, Hamburg Germany) equipped with a 63x or 100x/1.45 Oil TIRF objective and a Photometrics CoolSNAP HQ2 camera (Roper Scientific GmbH, Martinsried, Germany) and controlled by Visiview imaging suite (Visitron Systems).

To measure the cytoplasmic fluorescence signal intensity micrographs were processed using ImageJ version 1.48 (http://imagej.nih.gov/ij/). First, fluorescence signal intensity was measured from a randomly selected cytoplasmic region of interest within each spore. After this, the background intensity was measured by moving the same region of interest outside the spore where fluorescence was not affected by other fluorescent signals in the vicinity. Each background signal intensity measurement was subtracted from corresponding fluorescence signal intensity to calculate cytoplasmic fluorescence signal intensity.

2.8.2 Confocal microscopy

Confocal microscopic analysis was carried out using the Leica SP8 confocal laser-scanning microscope (Leica Microsystems (UK) Ltd, Milton Keynes, UK). Simultaneous red and green fluorescence of the lipophilic stains was excited by VS-LMS4 Laser-merge system with 488-nm/ 70 mW and 561nm/ 70 mW solid-state lasers (Visitron Systems GmbH, Puchheim,
Germany) systems. Micrographs were acquired the 63x and 100x oil objectives and analysed using Leica Application Suite X (Leica, UK) and/or ImageJ version 1.48.
Chapter 3

Exploitation of new drug resistance marker and non-homologous end joining mutants for functional analysis in Z. tritici
A substantial proportion of the text and data presented in this chapter were published in the research paper cited below.


Contributions to paper - Y.S. Sidhu planned and conducted all the experiments, and wrote the manuscript. K. Haynes and M. Csukai provided scientific advice. Y. K. Chaudhari provided technical assistance. K. Haynes, M. Csukai, N. J. Talbot, D. J. Studholme, J. Usher and T. C. Cairns provided critical feedback on the manuscript.
Abstract

The lack of techniques for rapid assembly of gene deletion vectors, paucity of selectable marker cassettes available for genetic manipulation and the low frequency of HR are the major constraints in the construction of gene deletion mutants of Z. tritici. To address these issues ternary vectors for AMT of Z. tritici were constructed to enable efficient vector construction by using the recombinational cloning in S. cerevisiae. The sulfonylurea resistance marker cassette \((p\text{ILV2:ILV2}^{\text{SUR}})\), which is based on mutated allele of the acetolactate synthase gene \(\text{ILV2}\) from M. oryzae, was established as a new dominant selectable marker for use in Z. tritici. To increase the HR frequency, Z. tritici strains deficient in the NHEJ pathway, which repairs the double-strand breaks in DNA, were constructed by inactivating the Z. tritici \(\text{KU70}\) and \(\text{KU80}\) genes. Targeted gene deletion frequency increased to more than 85% in both the Z. tritici \(\Delta\text{ku70}\) and \(\Delta\text{ku80}\) strains, compared to 6-10% observed in the WT strain IPO323. The \textit{in vitro} growth and \textit{in planta} pathogenicity of these \(\Delta\text{ku70}\) and \(\Delta\text{ku80}\) strains were comparable to the WT strain IPO323. Together these molecular tools are a significant addition to the molecular toolkit available for functional genomics analyses through targeted manipulations of the Z. tritici genome.
3.1 Introduction

STB disease of wheat is caused by *Z. tritici* and is a major constraint on global wheat production (Goodwin et al., 2011). Therefore, durable STB disease control strategies are urgently required. To develop such disease control measures, it is crucial to understand the molecular mechanisms that underlie the mode of pathogenesis adopted by *Z. tritici* (Orton et al., 2011).

In *Z. tritici*, targeted gene deletion is the main strategy for functional genetic analyses and has been used to characterise various genes (Appendix 1). These include genes that encode effectors (Motteram et al., 2009, Marshall et al., 2011), MAPKs (Mehrabi et al., 2006a, Mehrabi et al., 2006b, Mehrabi and Kema, 2006, Mehrabi, 2006) and ABC transporters (Zwiers et al., 2007, Zwiers et al., 2003). Currently, the AMT is the only available method for introducing transforming DNA into the *Z. tritici* genome (Zwiers and De Waard, 2001; Bowler et al., 2010). Similar to other ascomycete fungi such as *N. crassa* (Ninomiya et al., 2004), *S. nodorum* (Feng et al., 2012) and *M. oryzae* (Kershaw and Talbot, 2009), the gene targeting efficiency in *Z. tritici* is often very low and is influenced by the target loci (Bowler et al., 2010). This makes *Z. tritici* intractable to a medium or high-throughput reverse genetic screens.

In fungi, the HR frequency is influenced by several physical factors including the chromosomal position, chromatin structure and transcriptional state of the target genomic region (Ninomiya et al., 2004, Feng et al., 2012). In addition, the HR frequency is also affected by the NHEJ pathway, which repairs the breaks in double-stranded DNA (Walker et al., 2001). The NHEJ pathway competes with the HR pathway of DNA break repair (Carvalho et al., 2010). In several fungi, disruption of the NHEJ pathway leaves the HR pathway as the
dominant DNA break repair mechanism and as result the HR-mediated gene targeting efficiency increases (Ninomiya et al., 2004, Kershaw and Talbot, 2009). The eukaryotic NHEJ pathway is regulated by a protein complex comprising of the KU70–KU80 protein heterodimer and a DNA-dependent protein kinase catalytic subunit (Walker et al., 2001). Thus, loss of either or both the genes that encode these KU protein disrupts the NHEJ pathway (Ninomiya et al., 2004). The strains lacking the NHEJ pathway has been constructed for various filamentous fungi and this genetic tool has paved the way for high-throughput functional analysis in filamentous fungi (Carvalho et al., 2010).

The antibiotic resistance genes are routinely used as the positively selectable markers for genetic manipulations of various organisms (Yang and Naqvi, 2014). The limited availability of positive selection markers has withheld high-throughput functional analysis in Z. tritici. Currently, majority of the routine genetic manipulations in Z. tritici are carried out using three positively selectable marker genes, which confer resistance against different drugs (Payne et al., 1998, Bowler et al., 2010, Zwiers and De Waard, 2001, Kramer et al., 2009). These include the bar gene (isolated from Streptomyces hygroscopicus (Thompson et al., 1987)) that encodes phosphinothricin acetyltransferase and confers resistance against glufosinate ammonium; the nptII gene (isolated from E. coli) that encodes neomycin phosphotransferase II and confers resistance against geneticin (Bowler et al., 2010), and the hph gene (isolated from E. coli (Gritz and Davies, 1983)) that encodes hygromycin phosphotransferase and confers resistance against hygromycin B. In addition to these resistance genes, the carbendazim and carboxin resistance genes namely TUB1<sup>CBZR</sup> and SDHB<sup>H627Y</sup>, which are the mutated alleles of the Z. tritici gene TUB1 (encodes β-tubulin) and SDHB gene (encodes succinate dehydrogenase subunit B),
respectively, has also been reported as positively selectable markers for *Z. tritici* (Payne et al., 1998, Bowler et al., 2010). However, due to their potential off-target affects the $TUB1^{CBZR}$ and $SDHB^{H627Y}$ are rarely used in routine genetic manipulations of *Z. tritici*. The geneticin resistance marker cassette ($ptrpC:nptII$), comprising of the *Aspergillus nidulans* trpC promoter ($ptrpC$) and the *nptII* gene, has been used to inactivate the *Z. tritici KU70* gene (Bowler et al., 2010); therefore, only the *bar* and *hph* genes remain available for genetic manipulations in this $\Delta ku70$ strain. Thus, with availability of only these two resistance genes, it is currently impossible to construct triple gene deletion mutants in the *Z. tritici $\Delta ku70$* strain. The ability to inactivate multiple genes in the same genetic background has been particularly useful for characterising complex fungal virulence associated traits; for example the analysis secreted proteinases in the human pathogenic yeast *C. albicans* and zinc transporters in the human pathogenic fungus *A. fumigatus* (Amich et al., 2010, Watts et al., 1998). Therefore, it is crucial to increase the number of selectable markers available for genetic manipulations in order to facilitate the analysis of complex phenotypes in *Z. tritici*.

This study describes construction and utility of the *Z. tritici* $\Delta ku70$ and $\Delta ku80$ mutants in improving the gene targeting efficiency. In addition, establishment of the $pILV2:ILV2^{SUR}$ as a new dominant selectable marker cassette for genetic manipulation of *Z. tritici* is also reported. The *ILV2^{SUR}* gene is the mutated allele of the *M. oryzae ILV2* gene, which encodes acetolactate synthase, and confers resistance against the sulfonylurea herbicide chlorimuron ethyl (Valent and Chumley, 1991, Sweigard et al., 1994). Alongside, this study describes construction of the ternary vectors by modifying the *A. tumefaciens* binary vector backbone pCAMBIA-0380 for selection and replication in S.
cerevisiae. These ternary vectors allow single step cloning of multiple DNA fragments into vectors by using the HR mechanism of S. cerevisiae.

3.2 Materials and methods

General experimental procedures and materials used in this study are detailed in Chapter 2.

3.2.1 Growth conditions and culture media

Z. tritici strain IPO323 was used as the WT parental background, unless otherwise stated. All Z. tritici, S. cerevisiae, E. coli and A. tumefaciens strains were cultured as previously described in Chapter 2, section 2.1.

3.2.2 Nucleic acid manipulations

Details of genetic manipulation procedures used in this study are described in Chapter 2 section 2.2 and 2.3, unless otherwise stated. PCR primers used in this study are shown in Appendix 2.

3.2.3 Construction of the ternary vectors

The A. tumefaciens binary vector pC-HYG, which contained the hph gene under the control of ptrpC promoter (ptrpC:hph) on the transfer DNA (T-DNA) region, was modified to construct the pC-HYG-YR ternary vector (Addgene ID – 61765; https://www.addgene.org/61765/) which can replicate in S. cerevisiae. A 2483 bp DNA fragment containing the 2µ origin of replication and URA3 gene was amplified by PCR from the plasmid YEp24 (NEB) using primer pair 2µ-URA3-F and 2µ-URA3-R. PCR product was digested with restriction endonuclease SacII and ligated into the SacII digested pC-HYG using T4 DNA ligase (Chapter 2, section 2.3.1). The nucleotide sequence of
recombinant plasmids was verified Sanger sequence and the plasmid that imparted uracil auxotrophy in *S. cerevisiae* was designated as pC-HYG-YR.

The *hph* gene in pC-HYG-YR was replaced with the *bar* and *nptII* genes and the *pILV2:ILV2^SUR* cassette by cloning these markers into the pC-HYG-YR using yeast recombinational cloning (Chapter 2, section 2.3.2). The 1056 bp *nptII* gene was amplified from the plasmid pC-GEN (Motteram et al., 2009) using primer pair NPTII-F/R. The 552 bp *bar* gene was amplified from the plasmid pC-BAR (Kramer et al., 2009) using the primer pair BAR-F/R. Finally, the 2819bp *pILV2:ILV2^SUR* cassette was PCR amplified from pCB1532 (http://www.fgsc.net/fgn44/sweig.html) (Sweigard et al., 1994) using primer pair SUR-F/R. The pC-HYG-YR was digested with *PshAl* restriction endonuclease. PCR amplified resistance genes were mixed with the digested pC-HYG-YR and co-transformed into *S. cerevisiae*. Recombinant plasmids were rescued from uracil prototrophic *S. cerevisiae* strains and transformed into *E. coli*. Plasmids were isolated from *E. coli* and nucleotide sequence verified by Sanger sequence. Correct recombination placed the *bar* and *nptII* genes under control of the *ptrpC* promoter and the resulting cassettes were denoted *ptrpC:bar* and *ptrpC:nptII*, respectively. Recombinant ternary vectors containing *ptrpC:bar*, *ptrpC:nptII* and *pILV2:ILV2^SUR* cassettes were designated pC-BAR-YR (Addgene ID - 61766)(https://www.addgene.org/61766/), pC-G418-YR (Addgene ID - 61767)(https://www.addgene.org/61767/) and pC-SUR-YR (Addgene ID - 61768)(https://www.addgene.org/61768/), respectively (Figure 3.1 A).
Figure 3.1 A schematic map of the *A. tumefaciens* ternary vectors and a representation of vector construction by yeast recombinational cloning.

(A) Schematic map of *A. tumefaciens* ternary vectors pC-SUR-YR, pC-BAR-YR, pC-G418-YR and pC-HYG-YR containing marker cassettes (Marker) \( pLIV2:ILV2^{SUR} \), \( ptrpC:bar \), \( ptrpC:nptII \) and \( ptrpC:hpH \), respectively on the transfer DNA (TDNA) region between left border (LB) and right border (RB). \( Kan^R \) cassette confers resistance against kanamycin in *E. coli* and *A. tumefaciens* and the \( 2\mu \) origin of replication (\( 2\mu \) ori) and \( URA3 \) gene allow growth in *S. cerevisiae*. (B) Schematic showing workflow
involved in construction of a typical gene deletion vector using the yeast recombinational cloning (Figure modified Collopy et al., 2010). The left and right flanks of the target gene were PCR amplified from genomic DNA using primers that contain 30 nucleotides complementary to 5' and 3' of the Marker and 30 nucleotides complementary to 5' and 3' of digested backbone vector (Green and Brown). The in vivo homologous recombination in S. cerevisiae mediates the assembly of all the DNA fragments into a recombinant vector.

The vectors pC-G418-YR and pC-SUR-YR were used to construct the gene deletion vectors pC-G418-KU70-KO, pC-G418-KU80-KO and pC-SUR-KU80-KO for inactivation of the Z. tritici KU70 (Gene ID Mycgr3G85040; chromosome 3:729343-731703) and KU80 (Gene ID Mycgr3G40048; chromosome 4:2064048-2066336) genes, respectively. Briefly, 500 bp left flank (LF) (chromosome 3:728842-729342) and right flank (RF) (chromosome 3:731704-732204) regions of the KU70 gene were PCR amplified from genomic DNA of the WT strain IPO323, using primer pairs KU70-LF-F/R and KU70-RF-F/R, respectively. Subsequently, the PCR amplified KU70 LF and RF, and the EcoRi-HindIII digested pC-G418-YR vector were co-transformed into S. cerevisiae for yeast recombinational cloning. Similarly, 750 bp LF (chromosome 4:2063298-2064047) and RF (chromosome 4:2066337-2067086) of the KU80 gene were PCR amplified using the primer pairs KU80-LF-F/R and KU80-RF-F/R, respectively. The KU80 LF and RF were recombined into EcoRi-HindIII digested pC-G418-YR or pC-SUR-YR via yeast recombinational cloning. After nucleotide sequence verification, the recombinant vectors containing correctly recombined DNA fragments were designated pC-G418-KU70-KO, pC-G418-KU80-KO and pC-SUR-KU80-KO.

Similarly, 1,000 bp of LF and RF regions of the putative siderophore biosynthesis gene SidC (Gene ID Mycgr3G36951, chromosome 2:439283-453243), the putative α-(1,3)-glucan synthase gene AGS2 (Gene ID
Mycgr3G72646, chromosome 5:2631468-2639399) and the putative isocitrate lyase gene \textit{ICL1} (Gene ID Mycgr3G102083, chromosome 1:1020739-1022796) were PCR amplified using primer pairs SIDC-LF-F/R, SIDC-RF-F/R, AGS2-LF-F/R, AGS2-RF-F/R, ICL1-LF-F/R and ICL1-RF-F/R, respectively. PCR amplified LF and RF regions corresponding to each gene were recombined into the \textit{EcoRI-HindIII} digested pC-HYG-YR vector by yeast recombinational cloning. Sequence verified recombinant vectors used to inactivate the \textit{SidC}, \textit{AGS2} and \textit{ICL1} were denoted as pC-HYG-SidC-KO, pC-HYG-AGS2-KO and pC-HYG-ICL1-KO, respectively.

3.2.4 AMT of \textit{Z. tritici} and characterization of transformants

The gene deletion vectors pC-G418-KU70-KO, pC-G418-KU80-KO, pC-SUR-KU80-KO, pC-HYG-SidC-KO, pC-HYG-AGS2-KO, pC-HYG-ICL1-KO and pC-SUR-YR were introduced into \textit{Z. tritici} by AMT (Chapter 2, section 2.4). Putative \textit{Z. tritici} transformants were selected on BM agar containing 10 µg mL\(^{-1}\) chlorimuron ethyl (sulfonylurea hereafter) or YPD agar containing 200 µg mL\(^{-1}\) hygromycin or geneticin, and appropriate antibiotics.

To confirm the functionality of the \textit{pILV2:ILV2\textsubscript{SUR}} marker cassette in \textit{Z. tritici}, 15 independent sulfonylurea resistant transformants harbouring the ternary vector pC-SUR-YR were isolated and designated as the strains \textit{IPO323:ILV2\textsubscript{SUR}} 1–15. Genomic integration of the \textit{pILV2:ILV2\textsubscript{SUR}} in these strains was confirmed by diagnostic PCR using primers SUR-F and SUR-INT-R that were expected to amplify a single 558 bp product to indicate presence of this cassette. The putative \textit{Z. tritici} \textit{Δku70} (designated HLS1000) and \textit{Δku80} (designated HLS1001) strains, which harboured the plasmids pC-G418-KU70-KO and pC-G418-KU80-KO, respectively, were selected for resistance against
geneticin. Similarly, the sulfonylurea resistant putative Δku80 strains (designated as HLS1002) harbouring the vector pC-SUR-KU80-KO were also recovered. Inactivation of the KU70 and KU80 genes in these strains were analysed by diagnostic PCR using primer combinations KU70-EXT-F/KU70-INT-R/NPTII-INT-R, KU80-EXT-F/KU80-INT-R/NPTII-INT-R or KU80-EXT-F/KU80-INT-R/ILV2SUR-INT-R.

Southern blot analysis (Chapter 2, section 2.5) was performed to the confirm deletion of the KU70 and KU80 genes in the HLS1000, HLS1001 and HLS1002 strain. The genomic DNA isolated from the strains HLS1000, HLS1001 and the WT strain IPO323 was digested with restriction endonuclease PvuI and HindIII. Digested DNA was size fractioned by electrophoresis and transferred to blotting membrane. Membrane was probed with the 152 bp long DIG-dUTP labelled probe specific to the nptII gene, which was synthesized using primer pair Probe-F/R. In case of the KU70 and KU80 gene inactivation, this probe was expected to hybridize at 2409 bp and 2887 bp in the strains HLS1000 and HLS100, respectively. Similarly, genomic DNA isolated from the strain HLS1002 and the WT strain IPO323 was digested with BstEII and SphiI. A second DIG-dUTP labelled probe specific to RF region downstream of the KU80 gene, was synthesized using primer pair Probe2-F/R. This probe was expected to hybridize at 7241 bp to indicate the presence of the KU80 gene in the WT strain IPO323 and hybridization at 3792 bp was expected in case of the KU80 inactivation in the HLS1002 strain.

To compare gene-targeting efficiency in the HLS1000, HLS1001 and IPO323 parental backgrounds, the Z. tritici genes SidC, AGS2 and ICL1 were targeted by introducing the corresponding gene deletion vectors pC-HYG-SidC-
KO, pC-HYG-AGS2-KO and pC-HYG-ICL1-KO into each background. The hygromycin resistant transformants were recovered from each background and genomic DNA was isolated. Subsequently, diagnostic PCR was carried out using multiplexed primers. Primer combination SIDC-EXT-F/SIDC-INT-R/HPH-INT-R used to confirm the SidC deletion were expected to amplify a single 1735 bp product in case of the WT SidC gene, while a 2890 bp product was expected for its replacement by the ptrpC:hph cassette. Similarly, using primer combination AGS2-EXT-F/AGS2-INT-R/HPH-INT-R amplification of a 1200 bp product was expected for the WT AGS2 and 1900 bp product was indicative of its inactivation. Primers ICL-EXT-F/ICL-INT-R/HYGR-INT-R were expected to amplify 1400 bp product in case of the WT ICL1 gene and 2100 bp product to indicate replacement of the ICL1 gene by the ptrpC:hph cassette.

3.2.5 Drug sensitivity screen

To titrate the optimal inhibitory concentration of sulfonylurea, the strains IPO323 and strain IPO323:ILV2\textsuperscript{SUR} 1 were sub-cultured for ten rounds on YPD agar and subsequently spores used for drug sensitivity screens. For each strain, 10 µL aliquot of serially diluted spore suspensions (10\textsuperscript{6}, 10\textsuperscript{5} and 10\textsuperscript{4} spores mL\textsuperscript{-1} in sterile water) was spotted on BM agar containing sulfonylurea concentrations ranging from 0 to 12 µg mL\textsuperscript{-1}. Plates were imaged following 6 day incubation at 18 °C.

3.2.6 Characterisation of the \textit{Z. tritici} Δku70 and Δku80 strains

The \textit{Z. tritici} mutants HLS1000, HLS1001, HLS1002 and the WT strain IPO323 were screened to compare any phenotypic defects due to inactivation of the \textit{KU70} or \textit{KU80} genes. Mutagen sensitivity screen was conducted as described by Ninomiya et al. (2004). \textit{Z. tritici} spores were harvested from YPD
agar after 6 days of growth at 18 °C. Spores of each strain were spotted on SC agar amended with the various mutagens and plates were incubated for 6 days at 18 °C and image were acquired. For morphological analysis, each strain was cultured in SC, CD and MM-Zt broth for 72 h at 18 or 25 °C, 120 rpm and fungal biomass was microscopically analysed as described in Chapter 2, section 2.8.1. To analyse the impact of the *KU70* and *KU80* gene inactivation on virulence, the virulence assays were conducted as described in Chapter 2, section 2.6. Briefly, the second leaf of the 17 day-old wheat seedlings (susceptible cultivar Avalon) was inoculated with spores (10^7 spores mL\(^{-1}\) in inoculation solution) of each strain. Sterile inoculation solution was used for mock infections. Seedlings were maintained at 16:8 hour day: night cycles at 16 °C for 21 days and disease symptoms were analysed visually and by manually counting the pycnidial lesions on the leaf surface.

### 3.3 Results and discussion

#### 3.3.1 Yeast recombinational cloning for vector construction

This study aimed to increase the efficiency of vector construction for AMT of *Z. tritici*. To do so, four *A. tumefaciens* ternary vectors were constructed, which are compatible with yeast recombinational cloning that relies on the *in vivo* HR mechanism of *S. cerevisiae*. These ternary vectors pC-BAR-YR, pC-G418-YR, pC-HYG-YR and pC-SUR-YR harbour the *ptrpC:bar*, *ptrpC:nptII*, *ptrpC:hp* and *pILV2:ILV2\(^{SUR}\) resistance cassettes, which functionally confer resistance against glufosinate ammonium, geneticin, hygromycin and sulfonylurea, respectively, in *Z. tritici* (Figure 3.1 A).

Yeast recombinational cloning allows efficient cloning of multiple DNA fragments in a single step (Figure 3.1 B). This technique circumvents resource-
heavy conventional restriction ligation techniques, which involve multiple cloning steps (Kilaru and Steinberg, 2015). The optimised yeast recombination cloning protocol presented in the current study (Chapter 2 section 2.3.2) is highly reproducible and relatively inexpensive. This cloning methodology allows flexible replacement of various genetic elements such as promoters, terminators, genes, epitopes tags and resistance markers, which is invaluable feature for optimisation studies that involve testing of various genetic elements. The yeast recombinational cloning technique also allows in-frame fusion of genetic elements, which is often essential for gene expression studies and expression of fusion proteins for cell biology (Kilaru and Steinberg, 2015, Kilaru et al., 2015c). In this current research project, yeast recombinational cloning played a pivotal role by facilitating construction of various vectors used for AMT of *Z. tritici*, including the suite of 32 over-expression vectors (Sidhu et al., 2015b)(Chapter 4). Yeast recombinational cloning has been used elsewhere for high-throughput plasmid construction (Park et al., 2011a) and construction of the large DNA molecules ranging from 592 Kb (Gibson et al., 2008) to 2.3 Mb (Marschall et al., 1999). Thus, the yeast recombinational cloning protocol and the ternary vectors are an invaluable vector construction tool for genetic manipulation of *Z. tritici* and other organism amenable to AMT.

3.3.2 Functionality of the *pILV2:ILV2* <sub>SUR</sub> resistance cassette in *Z. tritici*

To establish the *pILV2:ILV2* <sub>SUR</sub> as new positive selection marker for *Z. tritici*, it was necessary to validate the sensitivity of *Z. tritici* to sulfonylurea. Spotting assay showed that the WT strain IPO323 was highly sensitive and fungal growth was completely inhibited at low sulfonylurea concentrations (2 µg mL<sup>-1</sup>) (Figure 3.2). However, for selection on cellophane discs, which are used
for AMT of Z. tritici, we observed that 10 µg mL⁻¹ sulfonylurea was needed to eliminate the background growth of Z. tritici (Data not shown). To confirm functionality of the plLV2:ILV2SUR marker cassette in Z. tritici, the vector pC-SUR-YR was introduced into the WT strain IPO323 by AMT and 15 sulfonylurea resistant transformants designated strain IPO323:ILV2SUR (1-15) were recovered. Diagnostic PCR was carried out on the genomic DNA isolated from these transformants and the strain IPO323 to confirm the genomic presence of the plLV2:ILV2SUR. As expected a single 558 bp fragment specific to the plLV2:ILV2SUR was amplified from the strains IPO323:ILV2SUR 1 - 15 but the product was absent in case of the WT strain IPO323 (Figure 3.2 A). Further, a single IPO323:ILV2SUR strain was sub-cultured for 10 rounds on non-selective medium and then spotted on BM agar containing 1–12 µg mL⁻¹ sulfonylurea (Figure 3.2 B). This strain retained drug resistance suggesting that the genomic integration of the plLV2:ILV2SUR marker cassette was mitotically stable even in absence of selection. This data demonstrated that the plLV2:ILV2SUR is functional as a dominant selection marker in Z. tritici.
Figure 3.1 Functionality of the $pILV2:ILV2^{SUR}$ resistance cassette in *Z. tritici*.

(A) Serially diluted spore suspensions ($10^6$, $10^5$ and $10^4$ spore mL$^{-1}$) of the wild type strain IPO323 and IPO323:$ILV2^{SUR}$ mutant strain, which harboured sulfonylurea resistance marker cassette $pILV2:ILV2^{SUR}$ were spotted on BM agar supplemented with various concentrations of sulfonylurea (chlorimuron ethyl) and plates imaged following 6 day incubation at 18 °C. (B) Agarose gel (1% w/v in TAE) image showing PCR amplification of a 558 bp PCR product which indicates that the $pILV2:ILV2^{SUR}$ cassette is present in the fifteen independent mutants IPO323:$ILV2^{SUR}$ 1–15 (Lane 01–15), but absence in the IPO323 (Lane 16/WT). Lane 17/V represents amplification of a 558 bp product from the pC-SUR-YR vector which carries $pILV2:ILV2^{SUR}$ cassette and was used as the positive control for PCR.

### 3.3.3 Inactivation of the *Z. tritici* KU70 and KU80 genes

A BLASTP search of the *Z. tritici* genome sequence, using the *N. crassa* mus-51 (NCBI accession AB177394) and mus-52 (NCBI accession AB177395) proteins as query, revealed the two *Z. tritici* proteins that were denoted as the KU70 (NCBI accession XM_003853648) and KU80 (NCBI accession...
XM_003853038), respectively. The 2361 bp long *Z. tritici KU70* gene has been previously characterised and mutants displayed increased the gene targeting efficiency (Bowler et al., 2010). The 2289 bp long *KU80* gene contains two introns. The KU80 polypeptide is 726 amino acids long and contains the KU70/KU80 N-terminal alpha/beta domain (amino acids 6-242), the KU70/KU80 beta-barrel domain (amino acid 241-457) and the C-terminal KU domain (amino acid 599-724). These conserved domains were also found in previously characterized fungal KU proteins (Ninomiya et al., 2004, Feng et al., 2012, Koh et al., 2014), which suggested that the KU80 is likely to be a component of the NHEJ pathway in *Z. tritici*.

To inactivate the *Z. tritici KU70* and *KU80* genes, respective gene deletion vectors pC-G418-KU70-KO, pC-G418-KU80-KO and pC-SUR-KU80-KO were introduced into the WT strain IPO323 by AMT. Diagnostic PCR was used to confirm the inactivation of the *KU70*, using the primer pairs KU70-EXT-F/KU70-INT-R and KU70-EXT-F/G418R-INT-REV (Figure 3.3 A). Out of 96 putative transformants tested, the amplification of a 2635 bp product indicated replacement of the *KU70* by the *ptrpC:nptII* marker cassette in three putative Δ*ku70* strains denoted as HLS1000 (1-3) (Figure 3.3 C). However, an additional 928 bp product, amplified using KU70-EXT-F/KU70-INT-R, in case of the HLS1000-2 and HLS1000-3 strains also suggested the presence of the WT *KU70* gene (Figure 3.3 C). The Δ*ku70* strain HLS1000-1 that lacked the WT *KU70* gene was designated as HLS1000.

The deletion of the *KU80* gene was confirmed by diagnostic PCR on another set of 96 putative mutants, harbouring the vector pC-G418-KU80-KO (Figure 3.3 B). Diagnostic PCR using the primer pair KU80-EXT-F/NPTII-INT-
REV amplified a single 3228 bp product in case of the three putative Δku80 strains denoted as HLS1001 (1-3), which indicated replacement of the KU80 gene by the ptrpC:nptII cassette (Figure 3.3 D). However, amplification of a 1619 bp PCR product, using primers KU80-EXT-F/KU80-INT-REV, from the strain HLS1001-3 suggested that the WT KU80 gene was present in this strain (Figure 3.3 D). The Δku80 strain HLS1001-1, which lacked the WT KU80 gene was designated as HLS1001. Southern blot analysis revealed that as expected, the ptrpC:nptII specific probe hybridized at single bands of 2409 bp and 2887 bp in the HLS1000 and HLS1001 strains, respectively, while no hybridization events were observed in case of the WT strain IPO323 (Figure 3.3 E F). This confirmed that the KU70 and KU80 genes had been inactivated without ectopic integrations.
Figure 3.3 Targeted deletion of the *Z. tritici KU70* and *KU80* genes.

The *Z. tritici KU70* and *KU80* gene deletion vectors pC-G418-KU70-KO and pC-G418-KU80-KO, respectively, were introduced into the WT strain IPO323 by AMT. (A and B) Schematics show strategy used for replacement of the *KU70* and *KU80* genes by the *ptrpC:nptII* marker cassette through homologous recombination between left flank (*KU70LF* or *KU80LF*) and right flank (*KU70RF* or *KU80RF*). The resulting strains that lacked the *KU70* and *KU80* genes denoted HLS1000 (Δ*ku70*) and HLS1001 (Δ*ku80*), respectively. (C and D) Image of agarose gel (2% in TAE (w/v)) shows the results of diagnostic PCR conducted on genomic DNA isolated from the putative HLS1000 and HLS1001 strains, and the WT strains IPO323. Primer pairs *KU70*-EXT-F/*KU70*-INT-R and *KU70*-EXT-F/NPTII-INT-R were expected to amplify 928 bp and 2635 bp products to indicate presence and inactivation of the *KU70* gene, respectively. Primer pairs *KU80*-EXT-F/*KU80*-INT-R and *KU80*-EXT-F/NPTII-INT-R were expected to amplify a 1619 bp product for the WT *KU80* gene and a 3228 bp product in case of its inactivation. (L) 1 kb DNA ladder. (E and F) Southern blot analysis on the genomic DNA isolated from the strains HLS1000, HLS1001 and IPO323. (E) DNA was digested with *Pvu*I and *Hind*III, size fractioned by electrophoresis (E). (F) The *ptrpC:nptII* specific probe (Probe in panel A and C) hybridised at 2409 bp and 2887 bp in the HLS1000 and HLS1001 strain confirming inactivation of the *KU70* and *KU80* genes, respectively, in these strains. Probe hybridization was not detected in the IPO323 strain.

To demonstrate utility of the *pILV2:ILV2SUR* marker cassette for gene deletion in *Z. tritici*, it was used to inactivate the *KU80* gene. For this, the vector
pC-SUR-KU80-KO, which harbours the \textit{pILV2:ILV2}^{\text{SUR}} flanked by 5’ and 3’ flanks of the \textit{KU80} gene, was introduced into the WT strain IPO323 (Figure 3.4 A). Colony PCR was carried out, using primers KU80-EXT-F/KU80-INT-R/SUR-INT-R to identify transformants carrying the replacement of the \textit{KU80} by the \textit{pILV2:ILV2}^{\text{SUR}} marker cassette. Out of 192 transformants that were screened, four showed a single 2490 bp product, which indicated inactivation of the \textit{KU80} gene (Figure 3.4 B). These putative \(\Delta ku80\) mutants were denoted as HLS1002 (1-4). Southern blot analysis showed that as expected the DIG-dUTP labelled probe hybridized at 3792 bp band, thus confirming the \textit{KU80} gene deletion without ectopic integration in the three HLS1002 strains (Figure 3.4 C). These experiments confirmed that the \textit{pILV2:ILV2}^{\text{SUR}} could be used as a positive selection marker for genetic manipulations of \textit{Z. tritici}.

Figure 3.4 Deletion of the \textit{Z. tritici} \textit{KU80} gene using the \textit{pILV2:ILV2}^{\text{SUR}} as a marker.

(A) Schematic of strategy used to replace the \textit{Z. tritici} \textit{KU80} gene by the \textit{pILV2:ILV2}^{\text{SUR}} cassette to construct the \(\Delta ku80\) strain HLS1002. Deletion vector pC-SUR-KU80-KO integrated into the \textit{KU80} locus of the WT strain IPO323 through homologous recombination between left (LF) and right (RF) flanks. (B) Image of an agarose gel (2% in TAE (w/v)) showing the results of diagnostic PCR carried out on the genomic DNA.
isolated from the HLS1002 (Lanes 2-4) and the IPO323 strain. Amplification of a 2203 bp product, using primer pair KU80-EXT-F/KU80-INT-R showed presence of the KU80 gene, while a 2490 bp product, using primer pair KU80-EXT-F/SUR-INT-R, confirmed the KU80 gene deletion. (C) Southern blot analysis performed on the genomic DNA isolated from IPO323 and the Δku80 null strains HLS1002 (Lanes 2-4). DNA was digested with BstEII-Sphi restriction endonucleases and probed using DIG-dUTP labelled probe (Probe in panel A) specific to the KU80 RF. Probe hybridized at 7241 bp indicating presence of the KU80 gene in the WT strain IPO323, while hybridization at 3792 bp indicated replacement of the KU80 gene by the pILV2:ILV2SUR marker cassette. (D) Virulence assay was conducted by inoculating spores of the strains HLS1002 and IPO323 on leaves of susceptible wheat seedlings (cultivar Avalon) and leaves were imaged after 21 days. Disease symptoms (including chlorosis, necrosis and pycnidia) induced by the strains IPO323 and HLS1002 were comparable with no observable differences. (E) Pycnidiospore containing cirri was released from the pycnidial lesions (arrows) indicating that the asexual reproduction was comparable between the strains HLS1002 and IPO323. Scale bar = 500 µm.

3.3.4 Characterization of the Z. tritici Δku70 and Δku80 strains

To test compare the impact of the KU70 and KU80 inactivation on the gene targeting efficiency, the Z. tritici genes SidC, AGS2 and ICL1 were inactivated in these background (Table 3.1). Using AMT, the deletion vectors pC-HYG-SidC-KO, pC-HYG-AGS2-KO and pC-HYG-ICL1-KO were individually introduced into the strains HLS1000, HLS1001 and the WT strain IPO323. Deletion of each gene was confirmed by diagnostic PCR on the genomic DNA isolated from 16 hygromycin resistant transformants recovered from each background (HLS1000, HLS1001 and IPO323). The deletion frequency of the SidC exceeded 90% in the strains HLS1000 and HLS1001, as compared to 6.25% observed in the WT IPO323 background (Figure 3.5 and Table 3.1). Similarly, the AGS2 and ICL1 genes were also inactivated at an elevated frequency exceeding 85% in the HLS1000 and HLS1001, as compared to less than 10% observed in case of the WT strain IPO323 (Table 3.1). The mutants lacking the Z. tritici genes SidC, AGS2 and ICL1 will be characterised in the follow up studies. These data reveal a dramatic increase in the gene targeting frequency as a result of inactivation of the Z. tritici KU70 and KU80 genes in the
strains HLS1000 and HLS1001, respectively. This is in agreement with previously reported increased gene deletion frequency that was achieved in an independently created \( Z.\ tritici \Delta ku70 \) strain (Bowler et al., 2010). In addition, the strain HLS1000 was also successfully used for high frequency targeted integration of gene overexpression constructs (Sidhu et al., 2015b, Cairns et al., 2015)(Chapter 4) and to replace the native promoter of the \( Z.\ tritici \beta-(1,3)\)-glucan synthase gene (Marchegiani et al., 2015)(Chapter 5).

Figure 3.5 High frequency targeted deletion of the \( SidC \) gene in the \( Z.\ tritici \Delta ku70 \) and \( \Delta ku80 \) strains.

(A) Strategy used to inactivate the \( Z.\ tritici \) \( SidC \) gene in the strains HLS1000, HLS1001 and the WT strain IPO323. The deletion vector pC-HYG-SidC-KO was introduced into each background by AMT. Vector integrated into the \( SidC \) locus through recombination between the homologous sequences the \( SidC \) LF and \( SidC \) RF and thus replaced the \( SidC \) gene with the hygromycin resistance cassette \( ptrpC:hph \).(B) Image of agarose gel (2% in TAE (w/v)) showing result of diagnostic PCR carried out, using primers \( SidC \) EXT-F/SIDC INT-R/HPH INT-R, on the genomic DNA isolated the putative \( SidC \) mutants recovered from each background (HLS1000, HLS1001 and IPO323). Amplification of a 1735 bp and 2890 bp products indicated the WT \( SidC \) gene and its replacement by the \( ptrpC:hph \) marker cassette, respectively. DNA isolated from the respective parental background was used as control (C). 1kb DNA ladder (L).
Table 3.1 Comparison of the gene targeting frequencies in the Z. tritici strains Δku70, Δku80 and the WT strain IPO323.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene ID</th>
<th>Size (bp)</th>
<th>Locus</th>
<th>Gene deletion frequency in Z. tritici strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>SidC</td>
<td>Mycgr3G36951</td>
<td>13961</td>
<td>Chromosome 2</td>
<td>HLS1000: 93.75% HLS1001: 100% IPO323: 6.25%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>439283-453243</td>
<td></td>
</tr>
<tr>
<td>AGS2</td>
<td>Mycgr3G72646</td>
<td>7932</td>
<td>Chromosome 5</td>
<td>HLS1000: 100% HLS1001: 87.50% IPO323: 0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2631468-2639399</td>
<td></td>
</tr>
<tr>
<td>ICL1</td>
<td>Mycgr3G102083</td>
<td>2058</td>
<td>Chromosome 1</td>
<td>HLS1000: 100% HLS1001: 93.75% IPO323: 6.25%</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>1020739-1022796</td>
<td></td>
</tr>
</tbody>
</table>

Due to disruption of DNA repair, the NHEJ mutants can become hypersensitive to DNA damaging mutagens (Hoff et al., 2010). To test whether inactivation of the KU70 and KU80 genes increased the mutagen sensitivity, the spores of the strains HLS1000 and HLS1001 were exposed to several DNA damaging mutagens. In line with the observations in N. crassa (Ninomiya et al., 2004), both the HLS1000 and HLS1001 showed mildly increased sensitivity to UV radiation, but not to chemical mutagens including methyl methane sulphate (MMS), hydroxyurea (HU) or benomyl (Figure 3.6 A). These compounds result in point mutations but not in double strand breaks in the DNA. Therefore it is not surprising that both the HLS1000 and HLS1001 strains are not hypersensitive to the tested mutagens. In addition, the microscopic analysis confirmed that the strains HLS1000 and HLS1001 showed no obvious morphological growth defects when cultured in the standard laboratory conditions (Figure 3.6 B). Similar to the WT strain IPO323, both the HLS1000 and HLS1001 strains...
displayed yeast-like budding spore growth in the SC medium, while the pseudohyphal and hyphal growth morphologies were predominant when these strains were cultured in nutrient limiting conditions of CDM and MM-Zt media (Figure 3.6 B).

Figure 3.6 Phenotypic screening of the *Z. tritici* strains Δku70 and Δku80.

(A) Serially diluted (10⁸ – 10⁴ spores mL⁻¹) 6 day-old spores of the *Z. tritici* strains HLS1000 and HLS1001, and the WT strain IPO323 were spotted on SC agar amended with either mutagen; methyl methanesulfonate (MMS) (1 µL mL⁻¹), hydroxyurea (HU) (1 mg mL⁻¹), benomyl (10 µL mL⁻¹) or exposed to ultraviolet (UV) radiation (350 J m⁻²). (B) The strains HLS1000, HLS1001 and IPO323 were cultured in the SC, CD or MM-Zt broth for 72 hours at 18-25 °C and 120 rpm. Micrographs were acquired in bright field channel at 63x magnification using the Olympus IX81 spinning disc microscope (Scale bar = 10 µm). (C) The virulence assay was conducted by inoculating spores (10⁷ spores mL⁻¹) of each strain on leaves of wheat seedlings (cultivar Avalon) and leaves were imaged 21 days after inoculation. The strains HLS1000 and HLS1001 were as pathogenic as the WT strain IPO323 and induced typical *Z. tritici* infection symptoms (necrosis, chlorosis and formation of the black/brown pycnidial lesions). (D) Quantitative analysis of infection (pycnidia cm⁻²) showed no significant difference in virulence of the strains HLS1000 (p = 0.25) and HLS1001 (p = 0.55) as compared to the WT strain IPO323. Pycnidia were counted manually and data was statistically analysed using analysis of variance (ANOVA).
To be useful as the parental background strains for functional analyses, it is imperative that deletion of the *KU70* and *KU80* genes or the marker cassettes (*ptrpC:nptII* and *pILV2:ILV2*$_{SUR}$) used for inactivation, do not detectably impact the *in planta* virulence during infection assays. To determine this, the wheat leaves (susceptible cultivar Avalon) were inoculated with the *Z. tritici* strains HLS1000, HLS1001, HLS1002 and the WT strain IPO323. Visual inspection of disease symptoms revealed no differences in initiation of chlorosis, necrosis and typical black brown pycnidial lesions on the leaves inoculated with any strain (Figure 3.6 C and 3.4 D). Quantification of the infection suggested that inactivation of *KU70* or *KU80* genes did not affect virulence and both the *Z. tritici* strains HLS1000 and HLS1001 were equally pathogenic as the WT parent strain IPO323 (Figure 3.6 D). Similarly, microscopic observation at 21 DAI revealed that pycnidiospore-bearing cirrhi was released from the characteristic pycnidial lesions formed on leaves inoculated by the strains HLS1002 and IPO323 (Figure 3.4 E). Similarly, the pycnidia on the leaves infected by the strains HLS1000 and HLS1001 also produced the pycnidiospores containing cirrhi, suggesting that inactivation of the *KU70* and *KU80* genes does not impair the asexual sporulation in *Z. tritici*. The comparable progression of infection symptoms and formation of pycnidiospores, the infectious propagules required for initiation of the STB infections (Orton et al., 2011), demonstrate that neither expression of the resistance cassettes and nor the inactivation of the *Z. tritici* *KU70* or *KU80* genes detectably affected the *in planta* virulence.

This study has demonstrated that the *pILV2:ILV2*$_{SUR}$ marker cassette facilitates robust selection without off target affects on growth and virulence of *Z. tritici*. In addition, the *pILV2:ILV2*$_{SUR}$ has no detectable nucleotide homology to the *Z. tritici* genome (analysed by NCBI MEGABLAST), which implies that
this marker cassette is unlikely to integrate at the off target loci due high HR frequency in these NHEJ mutants. The undesired phenotypes and off targeted integrations are typical issues associated with use of host derived genes such as the SDHB$^{H627Y}$ and the auxotrophic markers (de Gontijo et al., 2014, Sweigard et al., 1998, Hentges et al., 2005, Scalliet et al., 2012, Zhang et al., 2009). Based on the results presented in this study, it is proposed that the $pILV2:ILV2^{SUR}$ is an ideal selection marker for genetic manipulation of Z. tritici.

The Z. tritici KU70 and KU80 genes were deleted to increase the HR frequency and improve gene targeting. This objective was successfully accomplished as both the Z. tritici strains HLS1000 and HLS1001 facilitated targeted gene inactivation at higher efficiency (on average >90%) than the WT strain IPO323, for the three different genomic loci that were tested. The similar increase in the HR frequency was observed upon the deletion of the KU70 or KU80 genes in the other filamentous fungi such as N. crassa, M. oryzae, S. nodorum, and A. fumigatus (Feng et al., 2012, Villalba et al., 2008, da Silva Ferreira et al., 2006, Kershaw and Talbot, 2009). The elevated frequency of HR in the NHEJ deficient strains also confirms that NHEJ is the dominant pathway of double stranded DNA break repair in Z. tritici. This strengthens the proposition that in contrast to S. cerevisiae where DNA damage is predominantly repaired by the HR pathway (Rothstein, 1991) in most filamentous fungi the NHEJ pathway mediates the DNA break repair (Villalba et al., 2008; Ninomiya et al., 2004).

The HR frequency is influenced by the size homologous sequences present in the deletion constructs (Ninomiya et al., 2004, Feng et al., 2012). Due to small intergenic regions, we used relatively short homologous
sequences to inactivate the *KU70* and the *KU80* genes, which may have contributed to less than 5% gene targeting frequency in the WT strain IPO323. However, increase in the size of homologous sequences to 1000 bp, in the case of inactivating *SidC, ICL1* and *AGS2* genes, did not significantly increase the gene targeting frequency in the WT strain IPO323. This suggests that large homologous sequences were required to inactivate the *Z. tritici* genes in the WT strain IPO323. Similar to the *Z. tritici* strains HLS1000 and HLS1001, highly elevated gene targeting frequencies were achieved in the *N. crassa* NHEJ mutant strains by using 1000 bp flanking sequences (Ninomiya et al., 2004). This suggests that 1000 bp homologous sequences are sufficient to achieve the high frequency gene targeting in the NHEJ deficient *Z. tritici* strains.

The phenotypic characterization of the *Z. tritici* strains HLS1000 and HLS1001 confirmed that the *in vitro* growth and *in planta* virulence was comparable to the WT strain IPO323. These results suggest that the HLS1000 and HLS1001 strains are the ideal recipient backgrounds for rapid genetic manipulations in *Z. tritici*. The NHEJ deficient strains of *N. crassa* and *M. oryzae* have proved instrumental in dissecting the novel roles of various biological mechanisms including transcriptional regulators (Colot et al., 2006) and autophagocytosis (Kershaw and Talbot, 2009). The *N. crassa* NHEJ mutants also enabled construction of the whole genome deletion library (Collopy et al., 2010) which is an invaluable resource for molecular and cell biology in this model filamentous fungus (Fu et al., 2011, Berepiki and Read, 2013). Thus, the tools reported in this study should play an important role in furthering functional analysis in this economically important pathogen.
4.0 Conclusions

In this study, new tools were generated to facilitate high frequency targeted genetic manipulations and for high-throughput construction of *Z. tritici* mutant strains. The establishment of the *pILV2:ILV2SUR* marker cassette increases the number of positive selection markers available for use in this fungus. Along with the ternary vectors offering yeast recombinational cloning, these tools represent a significant addition to the molecular toolkit available for genetic manipulation of *Z. tritici*. AMT is widely used to genetically transform various fungi and plants (Michielse et al., 2005) and therefore these tools will also be useful for the wider plant pathology research community. The *Z. tritici* NHEJ deficient strains provide elevated frequency of targeted genome modification whilst maintaining the WT growth and virulence *in planta*. Taken together, these tools complement currently available techniques and are a major step towards making large-scale functional analysis projects feasible in *Z. tritici*. 
Chapter 4

A suite of Gateway® compatible ternary expression vectors for functional analysis in

*Z. tritici*
A substantial proportion of the text and data presented in this chapter were published in the research paper cited below.


Contribution to paper - Y.S. Sidhu planned and conducted all the experiments, and wrote the manuscript. K. Haynes, M. Csukai and Y. K. Chaudhari provided technical advice and assistance. K. Haynes, M. Csukai, J. Usher and T. C. Cairns provided critical feedback on the manuscript.
Abstract

Gene overexpression is a widely used functional genomics approach in fungal biology. However, to date it has not been established in Z. tritici, which is an important foliar pathogen of wheat. This study reports a suite of Gateway® recombination compatible ternary expression vectors for AMT of Z. tritici. The suite of 32 vectors is based on a combination of four resistance marker cassettes for positive selection against glufosinate ammonium, geneticin, hygromycin and sulfonylurea; three constitutive Z. tritici promoters (pZtATUB, pZtGAPDH and pZtTEF) and a nitrogen responsive promoter (pZtNIA1) for controlled gene expression. Half of the vectors facilitate expression of proteins tagged with C-terminal EGFP. All 32 vectors allow high frequency targeting of the expression cassettes into the KU70 locus and complement the KU70 gene deletion when transformed into the Z. tritici Δku70 strain, thus circumventing additional phenotypes that can arise from random integration. This suite of ternary expression vectors will be a useful tool for functional analysis through gene overexpression in Z. tritici.
4.1 Introduction

STB is a major foliar disease of wheat, which is caused by the ascomycete fungal pathogen *Z. tritici* (Suffert et al., 2013). Every year STB outbreaks reduce yields up to 50% (Eyal et al., 1987, Polley and Thomas, 1991), as such STB is globally regarded as an economically important cereal disease (Goodwin et al., 2011). The potential threat posed by STB has driven research into understanding the *Z. tritici* biology and molecular mechanisms underlying its mode of pathogenesis.

Functional genetic analysis in *Z. tritici* is predominantly restricted to targeted gene deletions using AMT (Zwiers and De Waard, 2001, Orton et al., 2011, Rudd, 2015). Over the last decade, this invaluable technique has provided new insights into molecular mechanisms that underpin growth, signalling, drug resistance and virulence (Gohari et al., 2014, Stergiopoulos et al., 2003, Zwiers et al., 2003, Motteram et al., 2009, Marshall et al., 2011, Mehrabi, 2006). However, the low HR frequency in *Z. tritici* is a major rate-limiting factor in generating gene deletion mutants (Chapter 3). In addition the indispensable essential genes, which encode potential drug targets, cannot be deleted (Oliveira-Garcia and Deising, 2013, Hu et al., 2007) and thus the function of their encoded proteins cannot be studied using gene deletion approach. Therefore, it is vital to develop new tools and establish alternative strategies for functional genomics in *Z. tritici*.

In the model organisms such as *S. cerevisiae* (Stevenson (Stevenson et al., 2001, Chua et al., 2006), *Schizosaccharomyces pombe* (Matsuyama et al., 2006) and the pathogenic yeast *C. albicans* (Chauvel et al., 2012), gene overexpression is widely used for functional characterisation. Gene
overexpression in these species was enabled by the development of high-throughput functional analyses tools such as expression vectors (Alberti et al., 2007, Chauvel et al., 2012, Nagels Durand et al., 2012) and genome-wide ORF libraries (ORFeome) (Chauvel et al., 2012, Cabral et al., 2012, Gelperin et al., 2005, Matsuyama et al., 2006). In addition to gene overexpression, these tools allow tagging of the ORFs with protein affinity tags or fluorescent protein coding genes for tandem-affinity protein purification and cell biological studies (Alberti et al., 2007). Expression vectors and ORFeome collections made it feasible to conduct large-scale cell biological studies involving fluorescent protein tagging of 6029 and 4431 ORFs in the model organisms S. cerevisiae and S. pombe, respectively (Huh et al., 2003, Matsuyama et al., 2006). Consequently, these tools have played a pivotal role in functional characterisation by revealing the novel biological information on subcellular protein localisation, transcriptional co-regulation as well as interactions between cytoskeletal organelles (Huh et al., 2003, Matsuyama et al., 2006). However, due to the lack of such tools in Z. tritici, overexpression and cell biology has not been used for functional genomics and studying the Wheat- Z. tritici interactions.

To address this bottleneck, we have constructed a suite of 32 expression vectors for gene overexpression and protein fusion to enhanced green fluorescent protein (EGFP) in Z. tritici. All vectors were constructed in the ternary vector pC-HYG-YR, which is compatible for replication and selection in E. coli, A. tumefaciens and S. cerevisiae (Sidhu et al., 2015a) (Chapter 3). The vectors can be propagated in E. coli, facilitate the yeast recombinational cloning in S. cerevisiae and can be introduced into Z. tritici using AMT. All vectors take advantage of the Gateway® recombination technology (Life Technologies Ltd, UK) and offer a choice of the three constitutive and one inducible Z. tritici
promoter, four positive selection markers, and come with, or without C-terminal EGFP fluorescent tag. Here we describe construction of these vectors and show their potential application for gene overexpression and protein fusion in Z. tritici.

4.2 Materials and methods

Growth conditions and molecular techniques used in this study are described in Chapter 2.

4.2.1 Strains and growth conditions

The Z. tritici Δku70 strain HLS1000 (Sidhu et al., 2015a)(Chapter 3) and the WT strain IPO323 were used as parental backgrounds. Z. tritici spores used for AMT and epifluorescence microscopy were harvested from YPD agar plates incubated at 15 °C for 6 days. The E. coli strain TOP10 ccdB\(^R\) (Life Technologies Ltd., UK) was used to propagate plasmids containing the Gateway\(^\circledR\) cassette; all other plasmids were propagated in the E. coli strain DH5\(\alpha\). The Z. tritici, A. tumefaciens, E. coli and S. cerevisiae cultures were grown as described in Chapter 2, section 2.1.

4.2.2 Nucleic acid manipulations

Standard protocols used for genetic cloning and nucleic acid manipulation are described in Chapter 2, section 2.2, unless otherwise stated. All primers used in this study are shown in Appendix 2.

4.2.3 Construction of entry clones

The donor vector pDONR207 (Life Technologies Ltd., UK) and standard Gateway\(^\circledR\) BP recombination cloning (Chapter 2, section 2.3.3) was used to
construct all the entry clones. Each ORF was PCR amplified using a forward primer containing the attB1 nucleotide sequence followed by the first 20 bp of the appropriate ORF and the reverse primer containing the attB2 sequence followed by the last 20 bp (without the stop codon) of the same ORF. To construct the entry clone pENTRY-EGFP, the 717 bp EGFP ORF was amplified from the plasmid pAG413GPD-ccdB-EGFP (Addgene plasmid - 14190) using primer pair attB1-EGFP-F/R. Similarly, the Z. tritici PRA1 (Mycgr3G42164) (chromosome 5: 224006–225198) and SOD1 ORFs (Mycgr3G103593) (chromosome 3: 677128–677962), which encode a putative pH responsive antigen and a Copper-Zinc superoxide dismutase, respectively, were amplified using primer pairs attB1-PRA1-F/R and attB1-SOD1-F/R. The PCR amplified EGFP, SOD1 and PRA1 ORFs were cloned into the donor vector pDONR207 and the resulting entry clones were denoted pENTRY-EGFP, pENTRY-SOD1 and pENTRY-PRA1, respectively.

4.2.4 Construction of destination vectors

Using the ternary vector pC-HYG-YR (Sidhu et al., 2015a)(Chapter 3), two expression vectors were engineered. These contain the KU70 left and right flanking regions (to target the Z. tritici KU70 locus), complete coding sequence of the KU70 gene (to complement the KU70 gene deletion in the Δku70 strain HLS1000), the ptrpC:hph marker cassette (for resistance against hygromycin in Z. tritici), the Z. tritici promoter pZtTEF (promoter of the putative transcription elongation factor coding gene TEF1 (Mycgr3G92705) to drive expression of the ORF), the attR1-ccdB-attR2 Gateway® cassette (to enable ORF cloning from the entry clones) and either a C-terminal stop codon or EGFP gene (to allow overexpression or protein tagging). To construct these two expression vectors,
the pC-HYG-YR was double digested with restriction endonucleases Ascl-BstEII and the 9193 bp backbone was gel purified. The genetic elements required for each vector were amplified by PCR using respective template and primers (Table 4.1). PCR products were gel purified to remove non-specific products. Components of each vector were mixed with the digested pC-HYG-YR backbone and co-transformed into S. cerevisiae for yeast recombinational cloning. Recombinant plasmids recovered from S. cerevisiae were propagated in the E. coli strain TOP10 ccdB<sup>®</sup> and subsequently their nucleotide sequence was verified using Sanger sequencing. The recombinant plasmid containing all components and Gateway<sup>®</sup> cassette with C-terminal stop codon was designated as pYSKH3, while the vector containing Gateway<sup>®</sup> cassette with C-terminal EGFP gene was designated as pYSKH15. (Figure 4.1A, Appendix 3).

**Table 4.1 Genetic elements used to construct the expression vectors.**

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<tr>
<th>Component</th>
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<th>Primer pair</th>
<th>Template</th>
<th>Notes</th>
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<td>LF-KU70-F/R</td>
<td>Genomic DNA from Z. tritici strain IPO323</td>
<td>Z. tritici KU70 gene and 1000bp left flank (KU70 LF). (chromosome 3 : 728343-731703)</td>
</tr>
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<td>pZtTEF-F/R</td>
<td>Genomic DNA from Z. tritici strain IPO323</td>
<td>Promoter of the putative Z. tritici translation elongation factor encoding gene (Mycgr3G92705) (chromosome 4:2027165-2028364)</td>
</tr>
<tr>
<td>pZtATUB</td>
<td>1149</td>
<td>pZtATUB-F/R</td>
<td>Genomic DNA from Z. tritici</td>
<td>Promoter of the putative α-tubulin encoding gene</td>
</tr>
<tr>
<td>Vector</td>
<td>Description</td>
<td>Use</td>
<td></td>
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<tr>
<td>--------</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>pZtGAPDH</td>
<td>1092</td>
<td>pZtGAPDH-F/R</td>
<td>Genomic DNA from <em>Z. tritici</em> strain IPO323</td>
<td>Promoter of the putative glyceraldehyde-3-phosphate dehydrogenase encoding gene (Mycgr3G99044) (chromosome 2:1142176-1143267)</td>
</tr>
<tr>
<td>pZtNIA1</td>
<td>1092</td>
<td>pZtNIA1-F/R</td>
<td>Genomic DNA from <em>Z. tritici</em> strain IPO323</td>
<td>Promoter of the putative nitrate reductase encoding gene (Mycgr3G111003) (chromosome 10:592340-593431)</td>
</tr>
<tr>
<td>Gateway® cassette</td>
<td>2018</td>
<td>ccdB-F/R</td>
<td>Plasmids DNA pAG423GPD-ccdB (Addgene plasmid 14150)</td>
<td>Gateway® cassette containing bacterial lethal gene ccdB flanked by attR1 and attR2 sites. (Alberti et al., 2007)</td>
</tr>
<tr>
<td>Gateway® cassette with C-terminal EGFP</td>
<td>2709</td>
<td>ccdB-F/R</td>
<td>Plasmids DNA pAG423GPD-ccdB-EGFP (Addgene plasmid 14198)</td>
<td>Gateway® cassette containing bacterial lethal gene ccdB flanked by attR1 and attR2 sites and C-terminal EGFP.</td>
</tr>
<tr>
<td>MCS</td>
<td>100</td>
<td>MSC-F/R</td>
<td>pC-HYG-YR</td>
<td>Multiple cloning site</td>
</tr>
<tr>
<td>KU70 RF</td>
<td>800</td>
<td>KU70-RF-F/R</td>
<td>Genomic DNA from <em>Z. tritici</em> strain IPO323</td>
<td>800bp right flank of <em>Z. tritici</em> KU70 gene (chromosome 3:731704-732503)</td>
</tr>
</tbody>
</table>

Subsequently, the Gateway® compatible expression vectors pYSKH3 and pYSKH15 were modified to construct the remaining 30 vectors (Appendix 3). Modifications included either replacement of the *ptrpC:hph* with one of the three marker cassettes namely *ptrpC:bar*, *ptrpC:nptII* or *pILV2:ILV2SUR*; and/or the pZtTEF promoter with one of the three *Z. tritici* promoters (Table 4.1; Figure 4.1) namely pZtGAPDH (promoter of the putative glyceraldehyde-3-phosphate dehydrogenase encoding gene Mycgr3G99044), pZtATUB (promoter of the *Z. tritici* α-tubulin gene TUB1) and pZtNIA1 (promoter of the putative *Z. tritici* nitrate reductase gene NIA1 Mycgr3G111003). To construct these 30 vectors, the pYSKH3 and pYSKH15 were digested with AatII-MfeI restriction enzymes.
endonucleases and the resulting 16,020 bp and 16,723 bp backbones respectively, were individually gel purified. The PCR amplified *Z. tritici* promoters and markers cassettes were individually mixed with the appropriate digested backbone and co-transformed into *S. cerevisiae* for yeast recombinational cloning. Recombinant plasmids were isolated from *S. cerevisiae* and propagated in the *E. coli* strain TOP10 *ccdB* and correct recombination of the cloned genetic elements was confirmed by DNA sequencing. Correct recombinant expression vectors were designated pYSKH1-32 (Appendix 3). Complete nucleotide sequence of these vectors are publically available through the following link https://www.dropbox.com/sh/k6df896pf660nk/AAB6XpWtiX5KoZaNPOm0rHB?dl=0#.

**4.2.5 Validation of the expression vectors**

Expression clones were constructed to determine levels of gene expression and targeting frequency of the expression cassettes into the mutated *KU70* locus in the Δ*ku70* strain HLS1000. Standard Gateway® LR recombination cloning (Chapter 2, section 2.3.3) was used to clone the *EGFP* ORF from the pENTRY-EGFP entry clone into the overexpression vectors pYSKH3, pYSKH7 and pYSKH11, which contain the *ptrpC: hph* marker cassette and the *pZtTEF*, *pZtGAPDH* and *pZtATUB* promoter, respectively, and the Gateway® cassette with C-terminal stop codon. The resulting expression clones were designated pYSKH34 (*pZtTEF:EGFP*), pYSKH35 (*pZtGAPDH:EGFP*) and pYSKH36 (*pZtATUB:EGFP*), respectively. Similarly, to confirm the functionality of vectors for protein tagging, the *SOD1* and *PRA1* ORFs were shuttled from the entry clones pENTRY-SOD1 and pENTRY-PRA1
into the expression vector pYSKH15, which contains the *ptrpC:aph* marker cassette, *pZtTEF* promoter and the Gateway® cassette with C-terminal *EGFP* tag. The resulting expression clones containing the *SOD1* and *PRA1* ORF were designated pYSKH46 (*pZtTEF:SOD1:EGFP*) and pYSKH47 (*pZtTEF:PRA1:EGFP*), respectively.

All expression clones were introduced into the Δ*ku70* strain HLS1000 by AMT (Chapter 2, section 2.4) and hygromycin resistant transformants were recovered after 12-14 days. Using the phenol chloroform method (Chapter 2; section 2.2.1), the genomic DNA was isolated from the parental background HLS1000, and the *Z. tritici* overexpression strains HLS1004 (Δ*ku70::KU70::ptrpC::aph::pZtTEF::EGFP*), HLS1005 (Δ*ku70::KU70::ptrpC::aph::pZtGAPDH::EGFP*), HLS1006 (Δ*ku70::KU70::ptrpC::aph::pZtATUB::EGFP*), HLS1007 (Δ*ku70::KU70::ptrpC::aph::pZtTEF::SOD1::EGFP*), and HLS1008 (Δ*ku70::KU70::ptrpC::aph::pZtTEF::PRA1::EGFP*), which harboured the expression clones pYSKH34, pYSKH35, pYSKH36, pYSKH46 and pYSKH47, respectively. Fungal DNA and primer pair pYSKH-INT-FWD/KU70-EXT-REV were used for diagnostic PCR to confirm integration of the expression cassettes at the target locus. These primers were designed to amplify a single 1500 bp product only if the expression clone integrated into the mutated *KU70* locus of the strain HLS1000 (Figure 4.1).

4.2.6 Western blotting

Western blot was carried out as previously described by (Chauvel et al., 2012). The *Z. tritici* strains HLS1004, HLS1005, HLS1006 and HLS1010 were cultured in YPD broth for 72 hours at 18 °C and 180 rpm. A 2 mL spore
suspension was centrifuged at 13000 rpm for 5 minutes and pellet was suspended in lysis buffer (0.1 M sodium hydroxide, 0.5M EDTA, 2% SDS, 2% β-mercaptoethanol) followed by a 5-minute incubation at 90 °C. A 10 µL aliquot of 4M acetic acid was added and spore lysate was neutralised at 90 °C for 5 minutes. Lysate was mixed with 50 µL loading dye (0.25 M Tris-HCl pH 6.8, 50% (v/v) glycerol, 0.05% (v/v) bromophenol blue) and loaded on 10% NuPage gel (Life Technologies Ltd., UK) for electrophoresis. After transfer to the nitrocellulose membrane, the EGFP protein was detected using Anti-Green fluorescent protein antibody (Sigma-Aldrich).

4.2.7 Microscopic analysis and quantification of fluorescence

Microscopic analysis and quantification of fluorescence was conducted as described in Chapter 2, section 2.8. Spores of the Z. tritici overexpression strains were exposed to a 488 nm solid-state laser at 20% intensity to excite the EGFP protein. Micrographs were acquired at 200 ms exposure using an inverted Olympus IX81 spinning disc microscope (Olympus, UK) and Visiview imaging suite (Visitron Systems, Germany). ImageJ version 1.48 (http://imagej.nih.gov/ij/) was used to measure the cytoplasmic fluorescence signal intensities. Each experiment was repeated twice and data from 100 individual measurements (50 per biological replicate/strain) were pooled for analysis.

4.3 Results and discussion

4.3.1 Overview of the Gateway® compatible expression vectors

Site-specific Gateway® recombination technology is based on the recombination mechanism of the bacteriophage lambda (Landy, 1989) and has
emerged as an efficient in vitro DNA cloning strategy (Hartley et al., 2000, Walhout et al., 2000, Alberti et al., 2007, Nagels Durand et al., 2012). This technique allows rapid cloning of PCR amplified ORFs to construct expression clones for gene overexpression, protein tagging or tandem affinity purification.

We constructed a suite of 32 Gateway® recombination compatible ternary expression vectors (designated pYSKH1-32; Appendix 3) in the A. tumefaciens backbone plasmid pC-HYG-YR using yeast recombinational cloning (Sidhu et al., 2015a, Collopy et al., 2010). In each expression vector, the transfer DNA (TDNA) region between the left (LB) and right (RB) border contains one of four resistance marker cassettes namely ptrpC:bar, ptrpC:nptII, ptrpC:hph and pILV2:ILV2SUR and one four Z. tritici promoters that control expression of the cloned ORF (Figure 4.1 A). These include three constitutive promoters pZtATUB, pZtGAPDH and pZtTEF of different strength, and an inducible promoter pZtNIA1, which is induced by nitrate and repressed by glutamic acid or other nitrogen sources (Marchegiani et al., 2015) (Chapter 5). Of the 32 vectors, 16 contain a direct C-terminal stop codon to express native proteins, and the other 16 vectors contain a C-terminal EGFP fluorescent tag that allows in-frame cloning of the ORF with the tag, separated by a short linker sequence. In S. cerevisiae this short linker sequence between the ORF and the tag does not generally interfere with folding and targeting of expressed proteins (Alberti et al., 2007). However, it is important to stress that in some cases the function of a protein may be adversely affected as a result of the linker sequence. In order to exploit the increased HR frequency of the Δku70 strain HLS1000 (Sidhu et al., 2015a)(Chapter 3), we have used this strain as a recipient background in our overexpression studies. We have included the entire coding sequence of the KU70 gene and the KU70 flanking sequences in
all the expression vectors. These flanking regions allow the targeting of expression cassettes into the mutated KU70 locus in the Δku70 strain HLS1000, while also complementing deletion of the KU70 gene at the same time. We have therefore created a set of Gateway® compatible ternary vectors that have one of four positive selection marker cassettes, one of four promoters, have or lack a C-terminal EFGP and are targeted to the KU70 locus in the strain HLS1000 (Figure 4.1 A).
Figure 4.1 Schematic map of the Gateway® compatible expression vectors and confirmation of their integration at target locus.

(A) The transfer DNA (TDNA) region between the left (LB) and right (RB) borders contains left the flank (KU70 LF), *Z. tritici* KU70 gene (KU70) and right flank (KU70 RF); one of the four drug resistance marker cassettes (Marker) namely *ptrpC*:bar, *ptrpC*:nptII, *ptrpC*:hph and *plLV2*:ILV2<sub>SUR</sub>; one of the four *Z. tritici* promoters (*pZt*) namely *pZtATUB*, *pZtGAPDH*, *pZtNIA1* and *pZtTEF*. Vectors for gene overexpression contain the Gateway® cassette (bacterial lethal gene *ccdB* flanked by the *attR*<sub>1</sub> and *attR*<sub>2</sub> sites) with no tag while vectors for protein tagging contain C-terminal *EGFP* tag, followed by terminator sequence (T). The 2µ origin of replication and *URA3* selection marker (2µ+URA3) allows growth in *S. cerevisiae*, kanamycin resistance gene (*Kan<sup>R</sup>*) and pBR322 origin of replication enable growth in *E. coli* and *A. tumefaciens*.

(B) Integration of the expression cassette into the mutated KU70 locus the strain HLS1000 through homologous recombination between the KU70LF and KU70RF sequences. (C) Image of agarose gel (2% (w/v) in TAE) shows the results of diagnostic PCR carried out on the genomic DNA using primer pair pYSKH-INT-FWD and KU70-EXT-REV. Amplification of a single 1500 bp product indicated integration of the expression cassette from the clone pYSKH34 (*pZtTEF*:EGFP) into the target locus in all six independent transformants of the *Z. tritici* overexpression strain HLS1004.

4.3.2 Confirmation of the functionality of expression vectors in *Z. tritici*

To confirm that the overexpression cassettes from the pYSKH34/35/36/46/47 expression clones integrated at the mutated *KU70* locus in the strain HLS1000, diagnostic PCR was carried out on the genomic DNA isolated from putative hygromycin resistant transformants of the *Z. tritici* overexpression strains. If the overexpression cassette integrates as expected, a 1500 bp PCR product will be amplified (Figure 4.2 B). All hygromycin resistant transformants of the *Z. tritici* strain HLS1004 (containing expression clone...
pYSKH34) amplified the expected 1500 bp band that was not seen in case of the Δku70 strain HLS1000 (Figure 4.2 C). Similar high frequency targeting was observed in case all other overexpression cassettes used to construct the overexpression strains HLS1004/5/6/7/8 (Data not shown). This confirms that the 1000 bp and 800 bp KU70 left and right flanking regions present in each expression vector were sufficient to facilitate efficient targeting of the overexpression cassettes into the mutated KU70 locus in the strain HLS1000.
Figure 4.2 Confirmation of functionality of the expression vectors for gene overexpression and protein tagging in *Z. tritici*.

(A) The *EGFP* expression driven by the constitutive *Z. tritici* promoters *pZtTEF*, *pZtGAPDH* and *pZtATUB* lead to strong cytoplasmic fluorescence signal in the *Z. tritici* strains HLS1004, HLS1005, and HLS1006 while only a weak background signal was recorded in the background strain HLS1000. *Z. tritici* spores were harvested from 72 hour old cultures grown at 18 °C 180 rpm and micrographs were acquired by using 488 nm solid-state laser and Olympus IX81 spinning disc microscope. (B) Western blot analysis using an *EGFP* specific antibody detected 32.7 kDa *EGFP* protein in the strain HLS1004, HLS1005, HLS1006 and the positive control strain HLS1010 in which *EGFP* was fused to the *trpC* promoter from *A. nidulans*. *EGFP* protein was not detected in the background strain HLS1000. (C) Quantification of *EGFP* fluorescence intensity in the strains HLS1004, HLS1005 and HLS1006 revealed that transcriptionally *pZtTEF* was the strongest *Z. tritici* promoter followed by *pZtGAPDH* and *pZtATUB*. (D) The SOD1-*EGFP* fusion protein in the strain HLS1007 was present uniformly in the cytoplasm while (E) the PRA1-*EGFP* fusion protein in the strain HLS1008 localized to certain subcellular compartments likely to be peroxisomes. (Scale bars represent 10 µm).

After confirmation of their targeting to the mutated *KU70* locus, the functionality of the *Z. tritici* constitutive promoters in the expression vectors was determined by analysing their ability to drive the expression of the heterologous fluorescent reporter gene *EGFP*. Hence, we quantified the fluorescence signal intensity of the *EGFP* protein in spores of the *Z. tritici* overexpression strains HLS1004, HLS1005, HLS1006 and the parental strain HLS1000. Visual analysis revealed strong cytoplasmic fluorescence signal in case of the strains HLS1004, HLS1005 and HLS1006, while only very weak fluorescence was observed in the strain HLS1000 (Figure 4.2 A).
Western blot analysis was carried out on the whole cell protein extracts prepared from the strains HLS1000, HLS1004, HLS1005, HLS1006 and the positive control strain HLS1010, in which the EGFP gene was expressed under the control of the ptrpC promoter. The EGFP protein specific antibody bound at a 32.7 kDa band in case of the strains HLS1004, HLS1005, HLS1006 and HLS1010, but not in the background strain HLS1000. (Figure 4.2 B) This suggested that indeed the cytoplasmic fluorescence signal originated from the EGFP protein as a result of the EGFP expression in the strains HLS1004, HLS1005 and HLS1006. To analyse strength of the constitutive promoters, the fluorescence intensity was compared between the strains HLS1004, HLS1005, HLS1006 and the parental strain HLS1000. The quantitative analysis revealed that pZtTEF was the strongest Z. tritici promoter followed by the pZtGAPDH and pZtATUB (Figure 4.2 C). These data illustrate that the Z. tritici promoters functionally drive expression of genes cloned into these expression vectors.

To confirm the application of the expression vectors for protein fusion, we constructed the Z. tritici strains HLS1007 and HLS1008, which contained the SOD1:EGFP and PRA1:EGFP transcriptional fusions, respectively, under the control of the pZtTEF promoter. The putative SOD1 gene encodes a 154 amino acid long peptide that contains a Cu–Zn superoxide dismutase domain and has 71% amino acid identity to the S. cerevisiae SOD1 (superoxide dismutase 1). In S. cerevisiae SOD1 is involved in oxidative stress resistance and localises to the cytoplasm and the nucleus (Tsang et al., 2014). Similarly global protein alignment revealed that the 301 amino acid long PRA1 peptide shared 48%, 35% and 29% identity to proteins encoded by the genes AspF2 (AFUA_4G09580) in A. fumigatus, ZPS1 (YOL154W) in S. cerevisiae and PRA1 in C. albicans. The AspF2, PRA1 and ZPS1 genes are induced under zinc
depletion and facilitate zinc uptake in *A. fumigates*, *C. albicans* and *S. cerevisiae* (Citiulo et al., 2012, Amich et al., 2010). BLASTP and Gene Ontology analysis revealed that the *Z. tritici* PRA1 contains an M35 Like superfamily domain for metallopeptidases and may be associated with peroxisomes (GO:00005371), vacuoles (GO:0000324) and/or cell wall (GO:0009277). Visual analysis revealed clear differences in the subcellular localization of the fusion proteins in the strains HLS1007 and HLS1008. The SOD1:EGFP fusion protein displayed strong cytoplasmic abundance while the PRA1:EGFP protein was restricted to specific subcellular organelles likely to be peroxisomes (Figure 4.2 D and E). This organelle specific localisation of PRA-EGFP is consistent with *C. albicans* in which PRA1 localises to peroxisomes (Marcil et al., 2008, Citiulo et al., 2012). These data show that the vectors can be used to tag and localize proteins for cell biology of *Z. tritici*.

The results presented in this study have demonstrated that the three constitutive promoters namely *pZtTEF*, *pZtGAPDH* and *pZtATUB* are functional in *Z. tritici*. Similarly, q-RT PCR and analysis of fluorescence intensity revealed that the nitrogen responsive promoter *pZtNIA1* was induced in the presence of nitrate and repressed upon exogenous application of glutamic acid, glutamine and ammonium (Marchegiani et al., 2015)(Chapter 5). These data imply that all four promoters can be used for gene overexpression in *Z. tritici*. In the model organism *S. cerevisiae* a large phenotypic screen revealed that overexpression of 371 ORFs caused deleterious growth defects and lethality (Gelperin et al., 2005). Alongside revealing their regulatory role, this observation provided preliminary evidence for putative essential genes (Gelperin et al., 2005). This is a one clear example of how gene overexpression will facilitate large-scale gene characterization in *Z. tritici*. One application of our overexpression strategy
would be to study the essential genes, which can be valuable drug targets and cannot be functionally characterized by targeted gene deletion (Hu et al., 2007). Therefore gene overexpression will play a pivotal role in studying the roles of essential genes in *Z. tritici*, which can be experimentally confirmed by a conditional promoter replacement approach (Marchegiani et al., 2015) (Chapter 5).

In addition to identification and characterisation of the essential genes in *Z. tritici*, overexpression using three constitutive promoters of different strength will enable elucidation of the complex phenotypes, which result from subtle changes in transcript abundance. For example, emergence of mutations in the promoter and subsequent overexpression of the *Z. tritici* sterol 14 α-demethylase gene *CYP51* (an azole fungicide target) resulted in 7 - 15 fold reduction in fungicide sensitivity in various isolates of *Z. tritici* (Cools et al., 2012). Thus, ability to overexpress the *Z. tritici* fungicide target genes at various strengths will be particularly useful in accessing the potential risk of fungicide resistance in the future.

In addition to gene overexpression, these data demonstrated that expression vectors facilitate gene tagging and expression of fusion proteins in *Z. tritici*. In *M. oryzae*, fluoresently tagged proteins have been crucial in untangling the role of effector secretion pathways and cytoskeletal remodelling during infection of rice (Giraldo et al., 2013, Dagdas et al., 2012). In the fission yeast *S. pombe*, fluorescent tagging vectors enabled subcellular localization of over 90% (4431 proteins) of the proteome and thus revealed novel components and regulators of cytoskeletal architecture (Matsuyama et al., 2006). Thus the ability to tag genes in *Z. tritici* will prove invaluable in studying the role of the *Z.*
*tritici* effectors in suppression of host immunity. As such this new capability in cell biology of *Z. tritici* will serve to study the role of cytoskeletal remodelling in morphological differentiation and stealth mode of pathogenesis deployed by *Z. tritici*.

**4.4 Conclusions**

In conclusion, we have constructed a suite of 32 ternary expression vectors potentially useful for gene characterisation in *Z. tritici* using gene overexpression and tagging. By complementing the existing tools such as the \( \Delta ku70 \) strain HLS1000, these vectors will facilitate rapid construction of the *Z. tritici* overexpression or tagged strains. These vectors will also enable the *Z. tritici* research community to exploit the full benefits of the *Z. tritici* ORFeome, which is being constructed at the University of Exeter. Thus together with existing tools and techniques, these expression vectors will serve as a robust platform for functional genomics through high-throughput *in vitro* screening on various biotic and abiotic stress conditions encountered by the pathogen during invasion of the host.
Chapter 5

Conditional gene expression and promoter replacement in *Z. tritici* using fungal nitrate reductase promoters
A substantial proportion of the text and data presented in this chapter were published in the research paper cited below:


**Contribution to paper** - Y. S. Sidhu planned and conducted the experiments and collected data presented in figures 5.1, 5.2 A and B and 5.3. E. Marchegiani provided the qRT-PCR data presented in Figure 5.2 C and assisted with writing the manuscript. K. Haynes and M. H. Lebrun provided technical advice and critical feedback on the manuscript.
Abstract

Studying essential genes in haploid fungi requires specific tools. Conditional promoter replacement (CPR) is an efficient method for verification of gene essentiality. This requires promoters that can be tightly controlled. To this end, the nitrate reductase promoters of *M. oryzae* (*pMoNIA1*) and *Z. tritici* (*pZtNIA1*) were cloned and tested for their ability to control gene expression in *Z. tritici*. Expression of the *EGFP* gene driven by the *pMoNIA1* or *pZtNIA1* was induced in presence of nitrate and repressed on glutamate (10-fold less than nitrate), as sole nitrogen source. Levels of differential expression were similar for both the promoters, demonstrating that the *Z. tritici* nitrogen regulatory network functions with a heterologous promoter in a manner similar to the native promoter. To establish the CPR strategy, the promoter of the *Z. tritici BGS1*, which encodes a β-(1,3)-glucan synthase, was replaced with the *pZtNIA1* using targeted sequence replacement. Growth of the *pZtNIA1:BGS1* CPR mutants was strongly reduced in the *pZtNIA1* repressing conditions, while their growth was similar to the parent strain in the inducing conditions. This differential growth phenotype illustrates that the *BGS1* is important for viability in *Z. tritici*. Data also showed that in the inducing conditions, the *pZtNIA1:BGS1* CPR mutants were hypersensitive to fungal cell wall stress agents. These nitrogen responsive promoters are therefore suitable for promoter replacement and controlled regulation of gene expression in *Z. tritici*. This molecular tool is a major step toward identifying novel fungicide targets in *Z. tritici*. 
5.1 Introduction

*Z. tritici* causes the STB disease of wheat which is a major threat to the global wheat production (te Beest et al., 2013). *Z. tritici* is characterised by a high genetic diversity and rapid adaptation to host plants and environment (Stukenbrock, 2013). Recent developments in the genomics of *Z. tritici* have significantly increased our understanding of the molecular interactions in the Wheat- *Z. tritici* pathosystem (Perez-Nadales et al., 2014). However, some molecular tools such as conditionally regulatable (inducible or repressible) promoters remain unavailable for controlling gene expression in *Z. tritici*. Regulatable promoters are a key tool for identification essential genes (Lamb et al., 2013, Delic et al., 2013, Hu et al., 2007) and controlled protein production (Helmschrott et al., 2013).

In filamentous fungi, expression of genes involved in nitrate (NO₃) assimilation is tightly controlled by exogenous nitrogen sources (Wong et al., 2008). This implies that promoters of these genes are ideal candidates for conditional regulation of gene expression. The genes involved in NO₃ utilization pathway have been extensively studied in the model filamentous fungus *A. nidulans* (Wong et al., 2008, Pateman et al., 1967). This metabolic pathway involves nine genes, which encode various enzymes or transporters (Wong et al., 2008, Bolton and Thomma, 2008). These include the nitrate transporters NtrA and NtrB, one nitrate reductase (NiaD), one nitrite reductase (NiiA), and five enzymes involved in biosynthesis of molybdate cofactor (Probst et al., 2014). In *Aspergillus* species, the *niaD*, *niiA* and *ntrB* genes are organized as a cluster (Slot and Hibbett, 2007), and the *niaD* and *niiA* are expressed under the control of a bidirectional promoter (Chang et al., 1996). Expression of the genes
which encode for these proteins involved in the NO$_3$ utilisation is regulated by two transcription factors (TFs), the nitrate-specific zinc finger TF NirA and a nitrogen-status sensing GATA TF AreA (Narendja et al., 2002, Burger et al., 1991).

In this study, the expression of the *Z. tritici* nitrate reductase gene *NIA1* was studied during growth on different nitrogen sources using qRT-PCR and transcriptional fusions using the *EGFP* gene as a fluorescent reporter. Alongside, the transcriptional regulation of the heterologous fungal nitrate reductase promoter (*pMoNIA1*) from *M. oryzae* was tested in *Z. tritici*. Subsequently, the promoter of *Z. tritici NIA1* (*pZtNIA1*) was used to verify essentiality of the *Z. tritici* β-(1,3)-glucan synthase gene *BGS1*. The β-(1,3)-glucan synthases encode for catalytic subunits, which are involved in the biosynthesis of fungal cell wall glucans and are essential for normal growth in several fungi including *A. fumigatus, Coccidioides posadasii* and *C. graminicola* (Firon and d’Enfert, 2002; Kellner et al., 2005; Oliveira-Garcia and Deising, 2013). Replacement of the *Z. tritici BGS1* promoter with the *pZtNIA1* illustrated that *BGS1* was important for growth and cell wall stress tolerance in *Z. tritici*.

5.2 Materials and methods

General growth conditions and methodologies used in this study are detailed in Chapter 2.

5.2.1 Strains, growth conditions standard nucleic acid manipulations

*Z. tritici* strain IPO323 was used as the WT background and the promoter replacement studies were conducted using the *Z. tritici Δku70* strain HLS1000 (Sidhu et al., 2015a)(Chapter 3). *Z. tritici* strains were cultured at 18 or 25 °C for
3 to 6 days in YPD or PD media. For nitrogen dependency, the minimal media containing 23 mM sodium nitrate (MM-Zt+NO$_3$), 23 mM ammonium tartrate (MM-Zt+NH$_4$), 23 mM glutamate (MM-Zt+Glu) or 23 mM glutamine (MM-Zt+Gln) were used. *A. tumefaciens*, *E. coli* and *S. cerevisiae* were cultured as described in Chapter 2, section 2.1.

### 5.2.2 Vector construction

Genetic cloning and all nucleic acid manipulations were conducted as described in Chapter 2, section 2.2, unless otherwise stated. PCR primers used in this study are shown in Appendix 2.

To construct the expression vector pELMA1, a 2903bp *EcoRI*-*XbaI* fragment containing the *EGFP* gene under control of the *pMoNIA1* promoter, was excised from the plasmid pSB4 (gifted by Marie-Josèphe Gagey, UMR MAP, Lyon, France) and cloned into the *EcoRI*-*XbaI* digested *A. tumefaciens* binary vector pBTH2 (Mullins et al., 2001). To place the *EGFP* gene under control of the *pZtNIA1* promoter, standard Gateway® LR recombination cloning (Chapter 2, section 2.3.3) was used to transfer the *EGFP* ORF from the Gateway® compatible entry clone pENTRY-EGFP into the ternary expression vector pYSKH27 (Sidhu et al., 2015b)(Chapter 4). Transfer DNA (T-DNA) region of the expression vector pYSKH27 carries the hygromycin resistance cassette *ptrpC: hph*, and the *pZtNIA1* promoter upstream of the Gateway® cassette containing *attR1-ccdB-attR2*. The resulting expression clone carrying the *pZtNIA1:EGFP* transcriptional fusion was designated pYSKH51.

To construct the promoter replacement vector pC-HYG-pZtNIA1-YR., the *pZtNIA1* promoter i.e. 1092 bp region immediately upstream of the *Z. tritici NIA1* gene was amplified using the primer pair pZtNIA1-FWD/REV and cloned into
the EcoRI-BamHI digested pC-HYG-YR vector (Sidhu et al., 2015a)(Chapter 3) using the yeast recombinational cloning (Chapter 2, section 2.3.2).

To replace the 500 bp \(p\text{TtBGS1}\) promoter (chromosome 11: 1,341,857-1,342,357) of the \(BGS1\) gene with the \(p\text{TtNIA1}\), the CPR vector pYSKH101 was constructed. For this, 1200 bp left flank (LF) region (chromosome 11:1,340,656–1,341,856) upstream of the \(p\text{TtBGS1}\) promoter was amplified using primer pair ZtBGS1-LF-F/R. Similarly, the 3000 bp right flank (RF) (chromosome 11:1,342,358–1,345,357), which comprises the first 3000 nucleotides of the \(BGS1\) coding sequence, was amplified using primers ZtBGS1-RF-F/R. Subsequently the LF and RF products were mixed with the EcoRI-BamHI digested pC-HYG-pZtNIA1-YR and co-transformed into \(S.\) \(cerevisiae\) for yeast recombinational cloning. Recombinant vectors were isolated from \(S.\) \(cerevisiae\) and propagated in \(E.\) \(coli\) and nucleotide sequences was analysed by Sanger sequencing before introduction into \(Z.\) \(tritici\).

5.2.3 AMT of \(Z.\) \(tritici\) and characterization of transformants

The vectors pELMA1, pYSKH51 and pYSKH101 were introduced into \(Z.\) \(tritici\) by AMT (Chapter 2, section 2.4). The vectors pELMA1 or pYSKH51 were introduced into the WT strain IPO323, while the pYSKH101 was introduced into the \(\Delta ku70\) strain HLS1000. Putative hygromycin resistant transformants were selected on YPD or MM-Zt+NO\(_3\) media amended with 200 \(\mu\)g mL\(^{-1}\) hygromycin. The strains harbouring the pELMA1, pYSKH51 and pYSKH101 vectors were denoted as the \(Z.\) \(tritici\) strains ZtpELMA1 (IPO323::pMoNIA1:EGFP), HLS1009 (IPO323::pZtNIA1:EGFP) and HLS1011 (HLS1000::pZtNIA1:BGS1), respectively.
Genomic DNA was extracted from the Z. tritici strains using the phenol chloroform method (Chapter 2; section 2.2.1). Integration of the pYSKH101 promoter replacement cassette at the BGS1 locus was confirmed by diagnostic PCR using the primer pair BGS1-EXT-FWD/-INT-REV. Primers were expected to amplify a 3790 bp or 5580 bp products to indicate presence of the pZtBGS1 or pZtNIA1 promoters upstream of the BGS1 gene, respectively.

5.2.4 RNA isolation and quantitative RT–PCR analysis

Total RNA was extracted from 5 µg of freeze-dried fungal mycelium using the RNeasy Plant Mini Kit (QIAGEN, France) according to the manufacturer’s instructions (Chapter 2; section 2.2.2). The cDNA was synthesised from 2.5 µg of total RNA using SuperScriptIII-Reverse transcriptase and oligo (dT) primers (Life technologies, UK). Real-time PCR was carried out using the gene specific primers, serially diluted cDNA, MESAGreen qPCRm MasterMix (Eurogentec, France) and CFX96 Real-Time System (BIORAD, France). Primer efficiency was tested using standard curves; primers with efficiency ranging between 95% to 100% and absence of non-specific products were used for real-time PCR. Relative gene expression levels were determined using the $2^{ΔΔCT}$ method (Livak and Schmittgen, 2001). The Z. tritici TUB1 (β-tubulin) gene was used as the reference. Experiments were carried out in biological triplicates and mean and standard deviations were calculated from relative gene expression.

5.2.5 Quantitative fluorescence assay

EGFP fluorescence was captured and quantified as previously described in Chapter 2, section 2.8. The Z. tritici strains ZtpELMA1 and HLS1009 were grown for 3 days at 18 °C and 180 rpm in MM-Zt+NO₃ or MM-Zt+Glu broth.
containing 0, 10, 20, 30, 40 and 50 mM sodium nitrate or glutamate, respectively. A 3 µL aliquot of spore suspension was spotted on 2% agarose (w/v in water) bed on a glass slide and exposed the 488 nm/50 mW solid-state laser (Visitron Systems, Munich, Germany). Micrographs were acquired using a 100x oil objective and at 200 ms exposure using the Olympus IX81 motorized inverted spinning disc microscope (Olympus, Hamburg Germany) controlled by Visiview imaging suite (Visitron Systems). Micrographs were analysed using ImageJ (version 1.48 http://imagej.nih.gov/ij) to calculate cytoplasmic fluorescence signal intensity (Chapter2, section 2.8). Average cytoplasmic fluorescence signal intensity for each condition was calculated from 100 individual measurements (50 per biological replicate) for each strain.

5.2.6 Phenotypic screen

Three independent transformants of the pZtNIA1:BGS1 CPR strain HLS1011 and the Δku70 strain HLS1000 were cultured in MM-Zt+NO_3 for 6 days at 18 °C and 180 rpm. Serially diluted spores were spotted on MM-Zt+NO_3 or MM-Zt+Glu agar media and incubated for 6 days at 18 °C. Similarly, serial dilutions of spore suspensions were also spotted on MM-Zt+NO_3 agar containing 100 µg mL^{-1} calcofluor white (CFW) or congo red (CR) and plates were imaged following 6 day incubation at 18 °C.

5.3 Results

5.3.1 Identification of the Z. tritici nitrate reductase NIA1 gene

BLASTP search of the Z. tritici genome database using the A. nidulans NiaD protein sequence (NCBI accession XP_658610.1) as query, identified a single uncharacterised protein (NCBI accession XP_003849160.1, E-value 0.0)
encoded by *Z. tritici* gene Mycgr3G111003 (chromosome 10: 592340-593431) later designated as *Z. tritici NIA1*. Global pairwise amino acid sequence alignment using EMBOSS Needle tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) revealed that the *Z. tritici* NIA1 shared 55.9% amino acid identity and 70% amino acid similarity with the *A. nidulans* NiaD. The *Z. tritici* NIA1 showed strong amino acid conservation with the nitrate reductase proteins from *N. crassa*, *M. oryzae*, *A. nidulans* and *A. fumigatus* (Figure 5.1 A). This suggested that the *Z. tritici* NIA1 could function as a nitrate reductase.

The 1172 bp intergenic region between the *Z. tritici NIA1* and the nitrite reductase gene *NII1* (Mycgr3G76215; NCBI accession XP_003848835.1) contains two putative binding sites (CTCCGHGG) for the *NIR1* like zinc finger transcription factor (NCBI accession XP_003849344.1), which is an ortholog of the *A. nidulans* NirA (Punt et al., 1995). This intergenic region also contains thirteen putative GATA binding sites for the NIR2 like transcription factor (NCBI accession XP_003852092.1), which is an ortholog of the *A. nidulans* AreA (Punt et al., 1995) (Figure 5.1 B). Furthermore, the *Z. tritici NII1–NIA1* intergenic region may be a bidirectional promoter, since the *NII1* gene is transcribed in opposite direction to the *NIA1*, and the *NIR1* and *NIR2* binding sites are found in this region. The *Z. tritici* genes encoding for putative nitrate transporters NTR1 (NCBI accession XP_003847717.1) and NTR2 (NCBI accession XP_003848428.1) are not located on chromosome 10 with the *NII1–NIA1* cluster. This organization differs from *A. nidulans* and *A. fumigatus*, which contain the nitrate transporter encoding genes adjacent to the *niaD* and *niiA* (Slot and Hibbett, 2007). Similar to *Z. tritici*, other fungi including *Dothistroma septosporum*, *Cladosporium fulvum*, *Septoria musicola* and *Mycosphaerella*
*fijiensis* have the bidirectional *NII1–NIA1* promoter and unlinked nitrate transporters. This organisation of the nitrate assimilation cluster in *Z. tritici* also differs from the dothideomycetes such as *Cochliobolus heterostrophus*, *L. maculans* or *S. nodorum*, which lack the *NII1–NIA1* gene cluster and divergent promoter.
5.1 Alignment of fungal nitrate reductase proteins and identification of transcription factor binding sites in the intergenic region between the *Z. tritici* NIA1 and NII1 genes.

(A) The nitrate reductase protein sequences from *Neurospora crassa* (Ncrassa; NCBI accession – XP_962069.1), *Magnaporthe oryzae* (Moryzae; NCBI accession – XP_003711915.1), *Zymoseptoria tritici* (Ztritici; NCBI accession – XP_003849160.1), *Aspergillus nidulans* (Anidulans; NCBI accession – XP_658510.1) and *Aspergillus fumigatus* (Afumigatus; NCBI accession - AAL85636.1) were aligned using the CLUSTAL Omega tool. Numbers show amino acid position in the protein sequence; asterisk (*) denotes identical amino acids; colon (:) shows conserved amino acids with strongly similar properties and period (.) shows conserved amino acids with weakly similar properties. (B) The nucleotide sequence of intergenic region (chromosome 10: 593432-595037) between *Z. tritici* NIA1 (Mycgr3G111003) and NII1 (Mycgr3G76215) genes. The predicted NII1 start codon CAT (reverse complement of ATG) is bold. The two putative binding sites namely Nir1(1) and Nir1(2) (positions -287 and -916), of the Zinc Finger transcription factor NIR1 (NCBI accession – XP_003849344.1) are underlined. The six putative binding sites namely Are1 (1) to Are1 (6) (positions -247, -345, -407, -830, -837 and -1154) of the GATA transcription factor Are1 (NCBI accession - XP_003849344.1) are underlined. The NIA1 expressed sequence tag (EST) starting at position 1012; NCBI EST accession - FM228048.1) is underlined. First 28 amino acids and corresponding nucleotides of the NIA1 gene are underlined; and two putative start codon (ATG) and translated methionine (M) amino acids are shown in bold.

5.3.2 Conditional gene expression in *Z. tritici*

To test functionality of the nitrate reductase promoter from *M. oryzae* (pMoNIA1) and *Z. tritici* (pZtNIA1) in *Z. tritici*, both promoters were fused to the fluorescent reporter gene *EGFP*. The plasmids pELMA1 (pMoNIA1:EGFP) and pYSKH51 (pZtNIA1:EGFP) were introduced into the WT strain IPO323 by AMT.
Both the *Z. tritici* strains HLS1009-1 and ZtpELMA1-1, which carried the plasmids pYSKH51 and pELMA1, respectively, showed a strong cytoplasmic EGFP fluorescence signal in MM-Zt+NO$_3$ media that contained sodium nitrate as a sole nitrogen source (Figure 5.2 A). On the other hand, growth on MM-Zt+Glu media containing glutamate as sole nitrogen sources caused strong reduction in the EGFP fluorescence (10-fold reduction compared to MM-Zt+NO$_3$) in both these strains (Figure 5.2 B). Quantitatively, fluorescence intensity was comparable between both these strains in each media. Further, different sodium nitrate concentrations (10, 20, 30, 40 and 50 mM) in MM-Zt+NO$_3$ did not significantly affect the fluorescence signal intensity in the strains HLS1009-1 and ZtpELMA1-1. This illustrated that both the *pMoNIA1* and *pZtNIA1* functionally regulated conditional gene expression in *Z. tritici*.

Next, the expression of *Z. tritici NIA1* gene was quantified in response to different nitrogen sources using qRT-PCR (Figure 5.2 C). The *Z. tritici NIA1* gene expression was 40 and 7 fold higher on MM-Zt+NO$_3$ media as compared YPD and PD, respectively (Figure 5.2 C). Replacement of nitrogen source from sodium nitrate in MM-Zt+NO$_3$ to glutamate (MM-Zt+Glu), ammonium (MM-Zt+NH$_4$) or glutamine (MM-Zt+Gln) reduced the *Z. tritici NIA1* expression by 9, 7 and 5-fold, respectively, as compared to MM-Zt+NO$_3$ (Figure 5.2 C). Further, qRT-PCR was used to quantify nitrogen source dependent regulation of the *pMoNIA1* promoter by analysing the *EGFP* gene expression the *Z. tritici* strain ZtpELMA1-1 (Figure 5.2 C). The *EGFP* gene expression was 18, 17 and 4-fold lower in MM-Zt+Glu, MM-Zt+NH$_4$ and MM-Zt+Gln, respectively, as compared to its expression in MM-Zt+NO$_3$ media. These data revealed that the *pMoNIA1* and *pZtNIA1* promoters showed relatively similar expression profiles in response to different nitrogen sources. Overall, the expression of the *EGFP*
gene driven by $pZtNIA1$ and $pMoNIA1$ was elevated in the presence of sodium nitrate as sole nitrogen source and repressed in presence of the reduced nitrogen sources including glutamate, ammonium and glutamine. Lastly, the expression profiles of both these promoters were independent of sodium nitrate concentrations.
Figure 5.2 Regulatable expression profiles of the nitrogen responsive promoters pMoNIA1 and pZtNIA1 in Z. tritici.

(A) Strong cytoplasmic EGFP fluorescence signal observed in spores of the strains ZtpELMA-1 (pMoNIA1:EGFP) and HLS1009-1 (pZtNIA1:EGFP) grown in MM-Zt+NO_3 media containing sodium nitrate as the sole nitrogen source. The Z. tritici strains were cultured in MM-Zt+NO_3 for 3 days at 18 °C and 180 rpm and micrographs were acquired in bright field (BF) and fluorescence/GFP (488 nm excitation) channels using 100x oil objective and 200 ms exposure. Scale bar = 20 µm (B) Nitrogen source dependent induction and repression of the EGFP fluorescence in the strains ZtpELMA-1, HLS1009-1 and the wild type strain IPO323 in MM-Zt+NO_3 (green bars) or MM-Zt+Glu (containing glutamate as sole carbon source)(grey bars). Bars show mean and error bars show standard deviations of the cytoplasmic fluorescence signal intensity calculated from 100 individual measurements. (C) The qRT-PCR based gene expression profiles of the Z. tritici NIA1 gene controlled by its native promoter pZtNIA1 (green bars) and the EGFP gene driven by pMoNIA1 (grey bars) in the strain IPO323. Total RNA was isolated from spores cultured in YPD, Potato dextrose (PD), MM-Zt+NO_3, MM-Zt+Gln (glutamine as a sole nitrogen source), MM-Zt+Glu and MM-Zt+NH_4 (ammonium as a sole nitrogen source) media. The cDNA was synthesized and qRT-PCR conducted using MESAGreen q-PCR MasterMix. Relative gene expression levels were calculated using the 2^{-ΔΔCt} method and the Z. tritici β-tubulin gene TUB1 as reference, Solid bars represent mean expression and error bars show standard deviations calculated from three biological replicates.

5.3.3 Conditional promoter replacement using the pZtNIA1 promoter

BLASTP search of the Z. tritici genome sequence, using the A. fumigatus β-(1,3)-glucan synthase Fks1p (NCBI accession XP_751118.1) (Beauvais et al., 2001) sequence as query, identified a single uncharacterized Z. tritici protein (NCBI accession XM_003848403) encoded by the gene Mycgr3G101655, later
designated as the *Z. tritici* **BGS1** gene. The *Z. tritici* **BGS1** shared 78% amino acid identity with the *A. fumigatus* Fks1p. To replace the **BGS1** gene promoter, **pZtBGS1**, with the **pZtNIA1**, the promoter replacement vector pYSKH101 was constructed using the backbone vector pC-HYG-pZtNIA1-YR, which contains the hygromycin resistance marker cassette *ptrpC:hph* and the **pZtNIA1** promoter (Figure 5.3 A). The vector pYSKH101 was introduced into the *Z. tritici* Δ**ku70** strain HLS1000 by AMT (Figure 5.3 B) and the putative hygromycin resistant **pZtNIA1:BGS1** CPR strains, designated as the *Z. tritici* strains HLS1011 (1-3), were selected from MM-Zt+NO₃ media. Fungal genomic DNA was isolated and diagnostic PCR was carried out using the **BGS1** gene promoter spanning primers **BGS1-EXT-FWD** and **BGS1-INT-REV**. As expected, these primers amplified a 5580 bp product in case of all three independent CPR strains HLS1011 (1-3) and a 3780 bp product in case of the background strain HLS1000 (Figure 5.3 C). This confirmed that the **pZtBGS1** was replaced by **pZtNIA1** in the three HLS1011 strains.

To down regulate the **BGS1** gene expression in the strains HLS1011 (1-3), these mutants and the Δ**ku70** strain HLS1000 were grown on the **pZtNIA1** inducing (MM-Zt+NO₃) and repressing (MM-Zt+Glu) conditions. In the inducing conditions, the growth of strains HLS1011 (1-3) was comparable to that of the HLS1000, at various spore concentrations (Figure 5.3 D). On the other hand, the strains HLS1011 (1-3) displayed a strong growth inhibition under repressing conditions at low spore concentrations (10⁶ and 10⁵ spores mL⁻¹), as compared to the strain HLS1000. At higher spore concentration (10⁷ spores mL⁻¹), the growth the HLS1011 (1-3) strains was mildly reduced than that of the HLS1000 (Figure 5.3 D). This leaky growth phenotype could be due to residual
transcription by the \( pZtNIA1 \) in repressing conditions, as observed in gene expression analysis through qRT-PCR (Figure 5.1 B and C).

Figure 5.3 Conditional promoter replacement and confirmation of essentiality of the \( Z. \) *tritici* \( BGS1 \) (\( \beta-(1,3) \)-glucan synthase) gene.

(A) Schematic map of the promoter replacement vector backbone pC-HYG-\( pZtNIA1 \)-YR, which contains the hygromycin resistance cassette \( ptrpC: \text{hph} \) and nitrogen responsive \( pZtNIA1 \) on the transfer DNA region between left (LB) and right (RB) border. The \( 2\mu \) origin of replication and \( URA3 \) marker (\( 2\mu \text{ori}+\text{URA3} \)) allows growth in \( S. \) *cerevisiae*; bacterial origin of replication pBR322 and pVS1, and the kanamycin resistance cassette \( \text{Kan}^R \) allow selection in \( A. \) *tumefaciens* and \( E. \) *coli*. (B) Construction of the \( Z. \) *tritici* strain HLS1011 (\( pZtNIA1:BGS1 \)) in which the promoter of \( BGS1 \) gene, \( pZtBGS1 \), was replaced with \( pZtNIA1 \) by introducing the vector pYSKH101 into the \( Z. \) *tritici* strain HLS1000 (\( \Delta ku70 \)). Promoter replacement was mediated through recombination between the homologous sequences \( LF/5' \text{UTR} \) and RF sequences present in the pYSKH101 and the \( BGS1 \) locus in the \( Z. \) *tritici* genome. (C) Agarose gel image (2% (w/v) in TAE) showing results of diagnostic PCR carried out using primer pair BGS1-EXT-FWD and BGS1-INT-REV, and the fungal genomic DNA isolated from...
the strains HLS1011. Amplification of 3790 bp and 5580 bp products indicates presence of the pZtBGS1 and pZtNIA1 upstream of the BGS1 gene, respectively. (D) Differential growth of the HLS1011 (1-3) strains on MM-Zt+NO\(_3\) and MM-Zt+Glu media. Spores harvested from 4 day-old MM-Zt+NO\(_3\) cultures grown at 18 °C and 180 rpm, were serially diluted (10\(^7\), 10\(^6\) and 10\(^5\) spores mL\(^{-1}\)) and spotted on each media and incubated for 6 days at 18 °C. (E) Hypersensitivity of the HLS1011 (1-3) strains to cell wall stress agents calcofluor white (CFW) and congo red (CR). Serially diluted spores suspensions were spotted on MM-Zt+NO\(_3\) containing 100 µg mL\(^{-1}\) CFW or CR and incubated for 6 days at 18 °C.

To study the role of BGS1 gene in cell wall stress tolerance, serially diluted spores of the pZtNIA1:BGS1 CPR strains HLS1011 (1-3) and the background strain HLS1000 were spotted on the pZtNIA1 inducing media MM-Zt+NO\(_3\) containing chemical cell wall stress agents calcofluor-white (CFW) or congo red (CR) (100 µg mL\(^{-1}\)). CFW and CR are toxic to fungi through their ability to bind to cell wall polysaccharides to destabilize cell wall structure (Ram and Klis, 2006). In the inducing conditions, the strains HLS1011 (1-3) displayed strong hypersensitivity to CFW and CR (at all spore concentrations) as compared to the background strain HLS100, which showed normal growth at these semi-lethal doses of CFW and CR. This screen illustrated that Z. tritici BGS1 gene played an important role in in vitro growth and tolerance of chemical cell wall stress.

5.4 Discussion and conclusions

This study has showed that the pZtNIA1 and pMoNIA1 promoters were useful to control gene expression of candidate genes in Z. tritici. Both these promoters lead to a high level of expression in the presence of sodium nitrate, while in the presence of glutamate, this expression is down-regulated by 18-fold according to qRT-PCR (pMoNIA1) and by 10-fold according to EGFP fluorescence assay (pMoNIA1 and pZtNIA1) (Figure 5.2 B and C). It should be
noted that glutamate was five times more efficient in repressing the expression of \textit{pMoNIA1} and \textit{pZtNIA1} than glutamine (Figure 5.2 C).

We have established a rapid promoter replacement strategy to exploit the \textit{pZtNIA1} for conditional gene expression of the native \textit{Z. tritici} genes. The conditional promoter replacement backbone vector pC-HYG-pZtNIA1-YR utilises the highly efficient yeast recombinational cloning for rapid construction of promoter replacement cassettes. The robustness of this CPR strategy is evident from successful replacement of the native promoter of the \textit{BGS}1 gene with the \textit{pZtNIA1} at a high frequency (100%) using the \textit{Δku70} strain HLS1000.

Growth of the \textit{pZtNIA1:BGS1} CPR mutant strains HLS1011 (1-3) was comparable to the background strain HLS1000 in inducing conditions, but strongly inhibited in repressing conditions and low inoculum. This suggests that the \textit{BGS}1 gene is important for normal growth of \textit{Z. tritici}. The orthologs of the \textit{Z. tritici} \textit{BGS}1 gene are essential for growth in several fungi (Beauvais et al., 2001, Firon et al., 2002, Oliveira-Garcia and Deising, 2013). In \textit{A. fumigatus}, Firon et al., (2002) were unable to recover viable haploid \textit{fksA} (also referred to as \textit{fks1} or \textit{bgs1}) deletion mutants from the heterozygous diploids carrying the \textit{fksA} deletion and WT alleles (Firon et al., 2002). Similarly, isolation of \textit{C. posadasii} homokaryon haploid strains carrying \textit{bgs1} deletion was not possible (Kellner et al., 2005). More recently, the \textit{GLS}1 gene (ortholog of the \textit{Z. tritici} \textit{BGS}1) in \textit{C. graminicola} was not inactivated and haploid mutants were not recovered following repeated attempts (Oliveira-Garcia and Deising, 2013). Thus, the \textit{BGS}1 gene would appear to be a widely conserved essential gene. However, recent data from \textit{A. fumigatus} contradicted this hypothesis, since viable haploid \textit{fksA} deletion mutants were obtained (Dichl et al., 2015). These \textit{fksA} mutants displayed severe morphological defects including abnormal and
reduced hyphal growth and hypersensitivity to cell wall stress agents (CFW and CR). Loss of the fksA also led to increase in cell wall chitin and galactosaminogalactans content and increase in these polysaccharides could be providing cell wall rigidity and partially compensating the loss of β-(1,3)-glucan (Dichtl et al., 2015). Down regulation of gene expression of the BGS1 orthologs by post transcriptional gene silencing led to reduced growth in C. graminicola (Oliveira-Garcia and Deising, 2013) and hypersensitivity to CFW in Metarhizium acridum (Yang et al., 2011). In C. graminicola and Z. tritici, down regulation the BGS1/GLS1 gene expression led to strong growth reduction suggesting a potential lack of compensatory mechanisms such as those observed in the A. fumigatus fksA deletion mutants (Dichtl et al., 2015).

The phenotypic screen revealed that the pZtNIA1:BGS1 CPR mutant strains HLS1000 (1-3) displayed a strong hypersensitivity to CFW and CR in inducing conditions. This hypersensitivity may be due to low the BGS1 gene expression in these mutants as compared to the background strain HLS1000, which subsequently lead to cell wall modifications that were not detrimental for growth but essential for cell wall stress resistance. The CFW and CR sensitivity assay also suggests that the use of conditional promoters with residual transcription in the inducing or repressing conditions would be invaluable for evaluating the relationship between mRNA levels and phenotypes for a given gene. Further investigations including quantitative expression analysis of the BGS1 gene in these CPR mutants will serve to identify mechanisms behind CFW and CR sensitivity. Future investigations must also interrogate whether overexpression of the BGS1 causes hyper-resistance to cell wall stress and reversal of the CR and CFW sensitivity? Equally, it will be interesting to study the virulence of BGS1 overexpressing strains. Transcriptional data shows that
at 4 DAI the \textit{BGS1} expression was 40\% lower than expression at 1 and 9 DAI (Rudd et al., 2015, Shetty et al., 2009). This down regulation of \textit{BGS1} could be an adaptive response to minimise elicitation of host defence responses because the \textit{β-(1,3)-glucan} from \textit{Z. tritici} cell wall acts as a PAMP and elicits PTI in wheat (Shetty et al., 2009). Oliveira-Garcia and Deising (2013) showed that down regulation of the \textit{GLS1/BGS1} gene in \textit{C. graminicola} delayed PTI elicitation in maize. Conversely, overexpression of the \textit{GLS1/BGS1} increased the \textit{β-(1,3)-glucan} content in the \textit{C. graminicola} cell wall, which lead to elevated expression of defence related genes including the PR and terpene synthase genes, and phytoalexin accumulation in maize (Oliveira-Garcia and Deising, 2013). Therefore, analysis of virulence of the \textit{Z. tritici BGS1} overexpressing strains would serve to elucidate whether cell wall modifications through increased \textit{β-(1,3)-glucan} content play a protective role against cell wall degrading enzymes produced by the host? In addition, the virulence phenotype of \textit{BGS1} overexpression strains would be invaluable in understanding the contribution of cell wall \textit{β-(1,3)-glucan} in modulation of PTI and subsequently the length of asymptotic phase of the STB infection?

Overall, the molecular tools developed in this study will be useful for identifying and characterising the essential genes in \textit{Z. tritici}. Such platforms are a prerequisite for developing new fungicides to control STB disease.
Chapter 6

The Glyoxylate cycle is an important virulence attribute in *Z. tritici*
Abstract

The *Z. tritici* genes *ICL1*, *MCL1* and *MLS1*, which encode isocitrate lyase (ICL), methylisocitrate lyase (MCL) and malate synthase (MLS) enzymes, respectively, were studied for their roles in fungal virulence. The ICL and MLS are key components of the glyoxylate cycle, which enables utilisation of non-fermentable carbon compounds as energy sources. The MCL is a key enzyme in the methylcitrate cycle, which serves to metabolise propionyl-CoA. Heterologous expression revealed that the *ICL1*, *MCL1* and *MLS1* functionally complemented loss of the orthologous genes in *S. cerevisiae*. Loss of the methylcitrate cycle caused no observable growth and virulence phenotypes in *Z. tritici*. Inactivation of the glyoxylate cycle severely affected pre-penetration spore-hyphal growth and subsequently virulence of the *Z. tritici* mutants on wheat leaves, illustrating that this pathway was required for normal infection related morphogenesis. More importantly, loss of the glyoxylate cycle was chemically complemented by exogenous glucose application. This result showed that the glyoxylate cycle plays an important role in pre-penetration morphogenesis possibly by enabling the fungus to produce energy through gluconeogenesis by utilising the stored reserves. These results clarify the ambiguity over the trophic nature of *Z. tritici* by revealing that this pathogen is not a hemibiotrophic pathogen, but a necrotrophic fungus that utilises internal reserves, potentially lipids or fatty acids, to fuel the morphogenesis for initiation of the STB disease cycle.
6.1 Introduction

Germination and host invasion are the most challenging stages during the infection cycle of a pathogen (Solomon et al., 2004). Intact plant surfaces present nutrient poor environments to the invading pathogens (Tucker and Talbot, 2001, Weber et al., 2001); therefore, pathogens have to overcome a period of nutrient deficiency until nutrition becomes available from alternative sources, such as cytosolic components following host cell death (Sanchez-Vallet et al., 2015, Keon et al., 2007). To deal with this, pathogens have evolved mechanisms including the ability to stockpile and metabolise long chain fatty acids to produce the energy required for growth during host invasion (Dunn et al., 2009).

One potential mechanism involved in releasing energy through fatty acid metabolism is the glyoxylate cycle. This anabolic pathway utilises the Coenzyme A (CoA) and acetyl-CoA, produced from β-oxidation of fatty acids, for synthesis of complex organic molecules (Lee et al., 2009). As a modified form of the tri-carboxylic acid (TCA) cycle, the glyoxylate cycle enables net carbon assimilation from simple two carbon compounds, thus bypassing the carbon dioxide generating steps present within the TCA cycle (Figure 6.1). Therefore, the glyoxylate cycle uses acetyl-CoA to replenish organic molecules; malate and succinate, which are required for production of glucose through gluconeogenesis. The glyoxylate cycle shares three enzymes in common with the TCA cycle, namely citrate synthase (CS), aconitase (ACN) and malate dehydrogenase (MDH); and involves two enzymes which are specific to the glyoxylate cycle, namely isocitrate lyase (ICL) and malate synthase (MLS) (Figure 6.1) (Dunn et al., 2009, Kondrashov et al., 2006).
Figure 6.1 Schematic representations of the glyoxylate cycle, the tricarboxylic acid (TCA) cycle and the methylcitrate cycle.

(A) The glyoxylate cycle specific steps are shown in red. The enzymes and genes (gene names from *S. cerevisiae*) involved in both pathways are shown. Figure taken from Lorenz and Fink (2001). (B) The methylcitrate cycle enzymes, which catalyse various steps involved in conversion of propionate to pyruvate, are shown in blue and the methylisocitrate lyase enzyme involved in the final step is highlighted in grey.

The glyoxylate cycle is a widely conserved pathway and found in a range of organisms including bacteria, fungi, archaea, nematodes, plants, amphibians, birds, fish and insects (Kondrashov et al., 2006). In plants, the glyoxylate cycle
underpins seed germination and growth in the absence of photosynthesis by enabling utilisation of stored lipid reserves as an energy source (Dunn et al., 2009). The glyoxylate cycle enzymes, ICL and MLS localise to peroxisomes; however, in the absence of a signal peptide essential for peroxisomal import, these enzymes can remain functional in the cytoplasm (Piekarska et al., 2008). The glyoxylate cycle has emerged as a major virulence-associated attribute due to its role in nutrition during the initial stages of fungal and bacterial pathogenesis (Wang et al., 2003, Lee et al., 2009, Ebel et al., 2006, Dunn et al., 2009). Survival of the human pathogenic bacterium Mycobacterium tuberculosis and the yeast C. albicans within macrophage cells (phagocytic cells forming the first line of defence in humans) required the glyoxylate cycle to be functional and its inactivation caused major reduction in virulence of both pathogens (McKinney et al., 2000, Lorenz and Fink, 2001). These virulence defects arose from an inability to catabolise lipids upon inactivation of either of the glyoxylate cycle enzyme coding genes, ICL1 and MLS1. Similarly, inactivation of the glyoxylate cycle through deletion of the ICL1 or MLS1 orthologs affected virulence of several phytopathogenic fungi including S. nodorum (Solomon et al., 2004), M. oryzae (Wang et al., 2003), L. maculans (Idnurm and Howlett, 2002), Colletotrichum lagenarium (Asakura et al., 2006) and G. zeae (Lee et al., 2009). Conversely, the glyoxylate cycle is dispensable for infection by the meningitis causing pathogen Cryptococcus neoformans and A. fumigatus that causes fatal invasive pulmonary aspergillosis in immunocompromised patients (Rude et al., 2002, Schobel et al., 2007).

Z. tritici causes the STB disease which is one of the most economically important diseases of wheat in the world (Goodwin et al., 2011). To initiate infection, the wind dispersed ascospores land on wheat leaves and germinate
into infectious hyphae, which invade the host through stomata and grow asymptomatically for 8 - 14 days within the nutrient poor apoplastic cavities of the mesophyll layer. It is unclear which sources and mechanisms allow *Z. tritici* to assimilate nutrients for pre-penetration morphogenesis and growth the extended asymptomatic phase of infection. Controversially, *Z. tritici* has been described as a hemibiotrophic pathogen, which implies that this fungus feeds on the host to acquire nutrients, despite the lack of evidence of biotrophic feeding or specialised structures required for such trophic relationship with the host (Orton et al., 2011). Furthermore, the composition of the nutrient poor apoplastic fluid remains unaltered during the asymptomatic phase (Shetty et al., 2009, Keon et al., 2007) suggesting that *Z. tritici* may not be relying on the apoplastic fluid as a sole nutrient source during early stages of infection. A recent study revealed that early stages of STB infection coincide with major transcriptional changes in expression of the *Z. tritici* genes involved in the glyoxylate cycle, and β-oxidation of fatty acids (Rudd et al., 2015). This suggested that lipid metabolism could be playing a key role in ATP (Adenosine triphosphate) generation and growth from the onset of STB infection (Rudd et al., 2015, Sanchez-Vallet et al., 2015, Orton et al., 2011). Transcriptional data from Rudd et al. (2015) also revealed that the genes involved in the methylcitrate cycle, which serves to metabolise propionyl-CoA produced during fatty acid β-oxidation, were up regulated early on in the infection process. One of these highly up regulated genes was the methylisocitrate lyase *MCL1*, which encodes a key enzyme in the methylcitrate cycle (Figure 6.1 B) and is known to regulate sexual reproduction in the wheat pathogenic fungus *G. zeae* (Lee et al., 2009). Moreover, a functional methylcitrate cycle is required for virulence in
the human pathogenic fungus *A. fumigatus* and *M. tuberculosis* (Eoh and Rhee, 2014, Ibrahim-Granet et al., 2008).

To address the long-standing question over the source and mechanism used by *Z. tritici* to acquire nutrition during the early stages of infection, the role of glyoxylate and methylcitrate cycles was investigated in this pathogen. The results revealed that the glyoxylate cycle is required for normal infection related growth and full virulence of *Z. tritici*, whereas the methylcitrate cycle is dispensable during infection of wheat. Additionally, results presented here show, for the first time, that lipid catabolism through gluconeogenesis could be providing the energy required by *Z. tritici* to invade, establish and induce acute STB disease symptoms within the host.

6.2 Materials and methods

Growth media compositions, environmental conditions and genetic manipulation techniques used in this study are described in Chapter 2, unless otherwise stated.

6.2.1 Growth conditions

The *Z. tritici Δku70* strain HLS1000 was used as the parental background for all the genetic manipulations. *S. cerevisiae* strain BY4741 used for yeast recombination cloning, the *E. coli* strains DH5α and TOP10 used to propagate the plasmids and the *A. tumefaciens* strain EHA105 used for the AMT of *Z. tritici*, were cultured as described in Chapter 2, section 2.1.

6.2.2 Construction of vectors

The *Z. tritici ICL1, MCL1* and *MLS1* genes were identified by a BLASTP search of the *Z. tritici* genome sequence and orthologous protein sequences
were downloaded from the NCBI repository and aligned using the CLUSTAL Omega tool. The MEGA6 suite was used for phylogenetic analysis.

For gene complementation studies in *S. cerevisiae*, the *Z. tritici* ICL1, MCL1 and MLS1 ORFs were placed under control of the constitutive *S. cerevisiae* promoter $P_{GPD}$ present in the expression vector pAG426GPD-ccdB (Addgene plasmid ID 14156; https://www.addgene.org/14156/), which contains URA3 selection marker to impart uracil auxotrophy in *S. cerevisiae* (Alberti et al., 2007). Using genomic DNA purified from the $\Delta ku70$ strain HLS1000 as template, the ICL1, MCL1 and MLS1 ORFs were PCR amplified using the primer pairs ICL1-F/R, MCL1-F/R and MLS1-F/R, respectively (Appendix 2). Each forward primer contained nucleotide sequences of the Gateway® recombination site attB1 followed by the first 20 bp specific to each ORF and the reverse primers included 20 bp before the stop codon of the specific ORF followed by the nucleotide sequence of Gateway® recombination site attB2. The PCR amplified ICL1, MCL1 and MLS1 ORFs were recombined into the donor vector pDONR207 using the Gateway® BP recombination cloning (Chapter 2, section 2.3.3) to construct the entry clones denoted as pENTRY-ICL1, pENTRY-MCL1 and pENTRY-MLS1, respectively. Sanger sequencing was used to verify the nucleotide sequence of each entry clone. To shuttle these ORFs from the entry clones into the expression vector, the entry clones pENTRY-ICL1, pENTRY-MCL1 and pENTRY-MLS1 were individually mixed with the expression vector pAG426GPD-ccdB in the presence of Gateway® LR Clonase™ enzyme to catalyse site-specific LR recombination cloning (Chapter 2, section 2.3.3). The resulting expression clones carrying the ICL1, MCL1 and MLS1 ORFs downstream of the $P_{GPD}$ promoter were designated as pGPD:ICL1, pGPD:MCL1 and pGPD:MLS1, respectively.
The gene deletion vectors pCSURICL1KO, pCBARMCL1KO and pCHYGMLS1KO used to inactivate the *Z. tritici* genes *ICL1*, *MCL1* and *MLS1* respectively, were constructed by yeast recombinational cloning (Chapter 2, section 2.3.2). Briefly, the 2100 bp long left flank (LF) and right flank (RF) regions flanking the *ICL1* gene were amplified from the genomic DNA by PCR using primer pairs ICL1-LF-F/R and ICL1-RF-F/R respectively. Similarly, 1500 bp LF and RF regions flanking the *MCL1* gene were PCR amplified using primer pairs MCL1-LF-F/R and MCL1-RF-F/R respectively. Finally, 1955 bp LF and 1995 bp RF regions flanking the *MLS1* gene were amplified by PCR using primer pairs MLS1-LF-F/R and MLS1-RF-F/R, respectively. The *A. tumefaciens* ternary vectors pC-SUR-YR (which contained the *pILV2:ILV2^{SUR}* marker cassette that confers resistance against sulfonylurea), pC-BAR-YR (which contained the *ptrpC:bar* marker cassette that confers resistance against glufosinate ammonium) and pC-HYG-YR (which contained the *ptrpC:aph* marker cassette that confers resistance against hygromycin) (Sidhu et al., 2015a) (Chapter 3) were double digested with restriction endonucleases EcoRI-HindIII. Subsequently, PCR amplified LF and RF corresponding to the *ICL1*, *MCL1* or *MLS1* genes were mixed with the digested vectors pC-SUR-YR, pC-BAR-YR or pC-HYG-YR, respectively, and co-transformed into *S. cerevisiae*. Recombinant plasmids were isolated from *S. cerevisiae* and propagated in *E. coli*. The nucleotide sequence of cloned DNA fragments in the recombinant vectors was analysed by Sanger sequencing.

### 6.2.3 Genetic transformation and analysis of transformants

The expression clones pGPD:ICL1, pGPD:MCL1 and pGPD:MLS1 were introduced into the *S. cerevisiae* strains Δicl1, Δicl2 and Δmls1 (background
strain BY4741; *MATa, his3, leu2, met15, ura3*), which lacked the *ICL1* (YER065C), *ICL2* (YPR006C: ortholog of *MCL1*) and *MLS1* (YNL117W) genes, respectively (Giaever et al., 2002, Giaever and Nislow, 2014). Standard lithium acetate and polyethylene glycol mediated genetic transformation method was used for transformation (Gietz and Woods, 2002) (Chapter 2, section 2.3.2.1) and uracil prototrophic transformants were recovered on SC-URA agar after 48 to 72 hour incubation at 30 °C. No growth was observed on the plates on which the negative control transformation mixtures (each background *S. cerevisiae* strain Δicl1, Δicl2 and Δmls1 transformed without complementation vector) were spread. The empty expression vector pAG426GPD-ccdB, without any ORFs, was introduced into the *S. cerevisiae* strains Δicl1, Δicl2 and Δmls1 and the resulting strains were designated as Δicl1::PGPD:ccdB, Δicl2::PGPD:ccdB and Δmls1::PGPD:ccdB, respectively. The *S. cerevisiae* strains Δicl1, Δicl2 and Δmls1 harbouring the expression pGPD:ICL1, pGPD:MCL1 and pGPD:MLS1, respectively, were designated as the strains Δicl1::ICL1 (Δicl1::PGPD:ICL1), Δicl2::MCL1 (Δicl2::PGPD:MCL1) and Δmls1::MLS1 (Δmls1::PGPD:MLS1), respectively.

To inactivate the *Z. tritici* ICL1, MCL1 and MLS1 genes, the gene deletion vectors pCSURICL1KO, pCBARMCL1KO and pCHYGMLS1KO were introduced into the strain HLS1000 using AMT (Chapter 2, section 2.4). The putative mutants lacking the ICL1, MCL1 and MLS1 genes were designated as the *Z. tritici* strains Δicl1 (Δicl1::pILV2:ILV2SUR), Δmcl1 (Δmcl1::ptrpC:bar) and Δmls1 (Δmls1::ptrpC:hph), respectively. The Δicl1 and Δmcl1 strains were selected on BM agar containing 10 µg mL⁻¹ sulfonylurea or containing 200 µg mL⁻¹ glufosinate ammonium, while the Δmls1 strain was selected on YPD agar containing 200 µg mL⁻¹ hygromycin, respectively. To construct the double
mutant strain Δicl1Δmls1 (Δicl1::pILV2::ILV2^{SUR}Δmls1::ptrpC:hph), AMT was used to introduce the MLS1 deletion vector pCHYGMLS1KO into the Δicl1 strain and hygromycin resistant (200 µg mL\(^{-1}\)) mutants were selected.
Figure 6.2 Inactivation of the Z. tritici ICL1, MCL1 and MLS1 genes.

The ICL1, MCL1 and MLS1 gene deletion vectors pCSURICL1KO, pCBARMCL1KO and pCHYGMLS1KO, respectively, were introduced into the Z. tritici strain HLS1000 (Δku70) by AMT. Each deletion vector integrated into corresponding Wild type ICL1/MCL1/MLS1 locus through homologous recombination between left (LF) and right (RF) flanking sequences present in each vector and the Z. tritici genome. Each resulting mutated ICL1/MCL1/MLS1 locus carried the pILV2:ILV2SUR, ptrpC:bar and ptrpC:aph marker cassettes in place of the ICL1, MCL1 and MLS1 genes in the mutant strains Δicl1, Δmcl1, Δmls1 and Δicl1Δmls1, respectively. Inactivation of each gene was verified by diagnostic PCR using specific multiplexed primers and the genomic DNA from the strains Δicl1, Δmcl1, Δmls1 and Δicl1Δmls1, and the background strain HLS1000 and the reference strain IPO323 as template. PCR results are shown as ethidium bromide stained agarose gel (2% w/v) images in panels A, B and C.

(A) Primers ICL1-EXT-F/ICL1-INT-R/SUR-INT-R were expected to amplify 2519 bp or 5089 bp products to indicate the presence of the ICL1 gene and its replacement by the pILV2:ILV2SUR marker cassette, respectively. (B) Primers MCL1-EXT-F/MCL1-INT-R/BAR-INT-R were expected to amplify 1794 bp and 2708 bp products to indicate the presence of MCL1 gene and its replacement by the ptrpC:bar cassette, respectively. (C) Primers MLS1-EXT-F/MLS1-INT-R/HYG-INT-R were designed to amplify the 2678 bp and 2930 bp products to indicate presence of the MLS1 gene and its replacement by the ptrpC:aph cassette, respectively.

Genomic DNA was isolated from all the independent transformants of the mutant strains Δicl1 (n=2), Δmcl1 (n=4), Δmls1 (n=2) and Δicl1Δmls1 (n=3), and the background strain HLS1000 and the parental wild type reference strain IPO323 using the standard phenol chloroform method (Chapter2, section 2.2.1). Multiplexed PCR strategy was used to identify the Z. tritici mutants lacking the corresponding target gene (Figure 6.2 A B C). Using a combination of primers ICL1-EXT-F/ICL1-INT-R/SUR-INT-R, the amplification of 2519 bp product indicated presence of the ICL1 gene in the strains HLS1000 and IPO323, while the 5089 bp product confirmed replacement of ICL1 by the pILV2:ILV2SUR marker cassette in all independent transformants of the strains Δicl1 and Δicl1Δmls1. Similarly, combination of PCR primers MCL1-EXT-F/MCL1-INT-R/BAR-INT-R yielded 1798 bp product, which showed presence of the MCL1 gene in the strains HLS1000 and IPO323; while amplification of the 2708 bp product confirmed replacement of the MCL1 by the ptrpC:bar marker cassette.
in all four independent transformants of the strain $\Delta mcl1$. As expected the primer pair MLS1-EXT-F/MLS1-INT-R/ HYG-INT-R amplified the wild type MLS1 specific 2675 bp product in case of the strains HLS1000 and IPO323, while amplification of the 2930 bp product confirmed the replacement of MLS1 by the pptrpC:hph marker cassette in independent mutants of the strains $\Delta icl1$ and $\Delta icl1\Delta mls1$. The diagnostic PCR showed that the ICL1, MCL1 and MLS1 genes were successfully inactivated in the strains $\Delta icl1.1$, $\Delta mcl1.1$, $\Delta mls1.1$ and $\Delta icl1\Delta mls1.1$, which were used for subsequent analysis.

6.2.4 Heterologous expression in S. cerevisiae

The S. cerevisiae strains $\Delta icl1$, $\Delta icl2$, $\Delta mls1$, $\Delta icl1::P_{GPD}:ccdB$, $\Delta icl2::P_{GPD}:ccdB$ and $\Delta mls1::P_{GPD}:ccdB$, $\Delta icl1::ICL1$, $\Delta icl2::MCL1$, $\Delta mls1::MLS1$ and the WT strain BY4741 were cultured for 14 hours at 30 °C and 180 rpm in SC media (containing 2% (w/v) glucose as sole carbon source). Cells were harvested by centrifuging at 13000 rpm for 5 minutes at room temperature, washed once with sterile water and cell concentration was adjusted to $10^7$ cells mL$^{-1}$ in water. Using a sterile loop, cells were streaked on SC agar containing 2% (v/v) ethanol or 2% (w/v) glucose as a sole carbon source. Serially diluted ($10^5$, $10^4$, $10^3$ and $10^2$ cells mL$^{-1}$) cells of the S. cerevisiae strains $\Delta icl2$, $\Delta icl2::P_{GPD}:ccdB$, $\Delta icl2::MCL1$ and the WT strain BY4741 were spotted on SC agar containing 1% (v/v) glycerol as a sole carbon source. Plates were imaged after 72 hour incubation at 30 °C.

6.2.5 Enzyme activity assay

Whole cell extracts were prepared from the Z. tritici strains $\Delta icl1.1$, $\Delta mls1.1$, $\Delta icl1\Delta mls1.1$ and the background strain HLS1000. Six-day-old spores ($10^8$) were inoculated in 50 mL MM-Zt broth containing either 2% (w/v) glucose
or sodium acetate, and cultured for 24 hours at 25 °C and 100 rpm. Fungal biomass was harvested and 200 mg was homogenised for 2 minutes in 1 mL buffer (containing Tris-HCl buffer (pH 7.5), 5 mM MgCl₂ and 0.8 mM EDTA) using 400 mg acid washed glass beads and the Fastprep®24 sample preparation system. Whole cell extracts were centrifuged at 4 °C for 30 minutes and 13000 rpm and supernatant was removed and maintained on ice until further use. Total protein content in the supernatant was quantified as described by (Bradford, 1976) using Coomassie brilliant blue G-250 dye and Bio-Rad protein assay kit. Absorbance was measured at λ₅₉₅ nm and total protein concentration relative to bovine serum albumin (0.1 – 1 mg mL⁻¹) was calculated from the standard curve. Measurements were carried out in technical triplicates and three biological replicates.

MLS enzyme activity was assayed at room temperature as described by (Smith et al., 2003) and Sigma-Aldrich protocol for Enzymatic assay of malate synthase (EC 4.1.3.2). Briefly, 500 µL of 50 mM imidazole buffer, 100 µL of 100 mM MgCl₂, 100 µL of 2.5 mM acetyl CoA, 100 µL of 10 mM glyoxalic acid, 100 µL of 2 mM 5,5'-Dithio-bis(2-Nitrobenzoic Acid (DTNB) and 100 µL of the supernatant from whole cell-extract or 100 µL of 50 mM imidazole buffer (for control) were added to a 2 ml cuvette and mixed by inversion. Absorbance at λ₄₁₂nm was measured for up to five minutes with one-minute intervals to obtain λ₄₁₂nm/minute from maximum linear rate for each sample and control. Units of MLS enzyme mg⁻¹ of extract were calculated as shown in equation 1.

ICL enzyme activity was assayed on crude cell extracts as described by (Lee et al., 2009, Dixon and Kornberg, 1959), using Sigma-Aldrich Assay for Enzyme activity of Isocitrate lyase (EC 4.1.3.1). Reactions were started by
adding 100 µL of the supernatant from whole cell-extract into a 2 mL cuvette containing 500 µL of 50 mM imidazole buffer, 100 µL of 50 mM MgCl₂, 100 µL of 10 mM EDTA, 100 µL of 40 mM phenylhydrazine and 100 µL of 10 mM isocitric acid. For blank or control reaction 100 µL of 50 mM imidazole buffer was used instead of the whole-cell extract. Cuvettes were mixed by inversion and absorbance at λ₃₂₄nm was measured at 1-minute intervals for up to 5 minutes and rate of change in absorbance over time was measured. ICL enzyme activity mg⁻¹ of extract were calculated as shown in equation 1. Both ICL and MLS enzyme activities were measured in technical triplicates and from three biologically replicated experiments.

**Equation 1 Calculation of MLS and ICL enzymes units in the extract.**

Absorbance at 324 nm or 412 nm per minute (A₃₂₄nm or A₄₁₂nm/min), Volume of the assay in mL (V), Dilution factor (DF), Extinction coefficient of DTNB at λ₄₁₂nm (13.6), Extinction coefficient of Phenylhydrazine at λ₃₂₄nm (16.8) and Volume of DTNB or Phenylhydrazine in the assay in mL (0.1).

\[
\text{MLS units mL}^{-1} = \frac{(A_{λ_{412nm}} \text{min of Sample} - A_{λ_{412nm}} \text{min of Control}) \times (DF) \times (V)}{(13.6) \times (0.1)}
\]

\[
\text{ICL units mL}^{-1} = \frac{(A_{λ_{324nm}} \text{min of Sample} - A_{λ_{324nm}} \text{min of Control}) \times (DF) \times (V)}{(16.8) \times (0.1)}
\]

\[
\text{MLS/ICL units mg}^{-1} \text{ of Protein} = \frac{\text{MLS/ICL units mL}^{-1}}{\text{Protein mg mL}^{-1} \text{ of extract}}
\]

**6.2.6 Virulence assay**

The virulence assays were conducted as defined in Chapter 2; section 2.6. Briefly, the adaxial side of second true leaves of 17 day-old wheat seedlings (cultivar Riband) were inoculated with a paintbrush using inoculum (10⁷ spores mL⁻¹) prepared from each mutant strain. Inoculated plants were
maintained at 16:8 hour day: night cycle at 18 °C and >85% relative humidity for 96 hours, followed by a reduction in humidity to 75% for up to 21 days. For exogenous glucose supplementation, a paintbrush was used to apply sterile 1 M glucose solution on the infected leaves after every 48 hours for up to 21 DAI. Inoculated leaves were visually inspected for symptoms of infection and imaged at 21 DAI to count pycnidia cm\(^{-2}\). Each strain was inoculated in five technical replicates and pathogenicity assays were biologically replicated at least three times.

6.2.7 Epifluorescence microscopy and analysis of cellular lipids

Microscopic analysis was conducted as described in Chapter 2, section 2.8. Briefly, spores (10\(^5\) spores) of each *Z. tritici* strain were inoculated on an agarose cushion (2% (w/v) in water) on a sterile glass slide and incubated at 25 °C for up to 48 hours to allow spore elongation. For lipid staining, 10 µM solution of BODIPY and/or 1 µM Nile red (1mM stock in DMSO) was added to the agarose cushion or leaf sections mounted on a glass slide and incubated for 5 minutes at room temperature in the dark. To visualize lipids, fluorescence was excited at \(\lambda_{488\text{nm}}\) using the solid-state argon laser and emission captured at \(\lambda_{510-515\text{nm}}\) for BODIPY and at \(\lambda_{560-565\text{nm}}\) for Nile red. Micrographs were acquired using 63x or 100x oil objective and the Leica TCS SP8 confocal microscope and spore length was quantified using ImageJ software.

To induce hyphal growth, the spores of each *Z. tritici* strain were cultured for 12 days at 25 °C on wheat leaf agar plates (400 grams blended wheat leaves in water to make 1 litre). Wheat leaf agar was expected to be abundant in host cytosolic contents such as sugars, which were released from autoclaved leaves. Therefore, this agar medium was preferred over the MM-Zt minimal
media in order to mimic the most nutritionally abundant environments faced by Z. tritici during the course of STB infection. Micrographs were acquired as described above.

6.3 Results

6.3.1 Identification of the Z. tritici ICL1, MCL1 and MLS1 genes

By using the BLASTP search and the S. cerevisiae ICL1 (YER065C; NCBI accession AAB64601) as query, two Z. tritici genes Mycgr3G102083 (E value 1.03E^{-168}) (Chromosome 1:1020739-1022796) and Mycgr3G83726 (E value 6.32E^{-139}) (Chromosome 1:2611979-2613717) were identified as the putative ICL1 genes in the Z. tritici genome. The 537 amino acid long Mycgr3G102083 peptide (NCBI accession XP_003855965) and the 560 amino acid long Mycgr3G83726 peptide (NCBI accession XP_003856249) shared 61.1% and 49.3% identity with the S. cerevisiae ICL1, respectively. Both of these Z. tritici peptides shared 45% and 60.3% amino acid identity and similarity, respectively, with each other. Gene Ontology (GO) analysis showed that both these proteins had isocitrate lyase activity (GO accession GO:0004451). Phylogenetic analysis revealed that the Mycgr3G102083 (denoted as ICL1) protein clustered on the same sub-clade as the characterised isocitrate lyase proteins from other fungi including M. oryzae (Wang et al., 2003), N. crassa (Gainey et al., 1992), C. albicans (Lorenz and Fink, 2001), G. zeae (Lee et al., 2009), A. fumigatus (Schobel et al., 2007, Ebel et al., 2006), L. maculans (Idnurm and Howlett, 2002), A. nidulans (Amor et al., 2000) and S. cerevisiae (Schöler and Schüller, 1993) (Figure 6.3 A). The Mycgr3G83726 (denoted as MCL1) protein clustered with the characterised and uncharacterised methylisocitrate lyase proteins from A. fumigatus, A. nidulans
(Brock, 2005), G. zaeae (Lee et al., 2009), S. cerevisiae (Luttik et al., 2000) and other organisms (Figure 6.3 A). Further, protein sequence alignment showed that as observed for other isocitrate lyase proteins, the Z. tritici ICL1 contained the typical isocitrate lyase specific substrate-binding site (QFITLAG) with the conserved phenylalanine (F) and threonine (T) amino acid residues. In the Z. tritici MCL1, the substrate-binding site (QLISLAG) contained the amino acids leucine (L) and serine (S) instead of phenylalanine and threonine (Fig 6.1b). This amino acid replacement is a conserved feature of the fungal methylisocitrate lyase proteins and allows for the acceptance of the additional methyl-group within the substrate-binding site (Muller et al., 2011).
Figure 6.3 Phylogeny and sequence analysis of the *Z. tritici* ICL1 and MCL1 proteins.

(A) The evolutionary history of the isocitrate lyase and the methylisocitrate lyase proteins was inferred using the Maximum Likelihood and the bootstrap consensus tree inferred from 1000 replicates is shown. Numbers next to each branch show the percentage of replicate trees in which the associated taxa clustered together. Protein sequences were retrieved from NCBI database and examined using the MEGA6 suite. The isocitrate lyase and methylisocitrate lyase protein sequences from the following organisms were included; *A. fumigatus*, *Aspergillus fumigatus*; *A. nidulans*, *Aspergillus nidulans*; *C. albicans*, *Candida albicans*; *C. lagenarium*, *Colletotrichum lagenarium*; *C. neoformans*, *Cryptococcus neoformans*; *C. tropicalis*, *Candida tropicalis*; *G. zeae*, *Gibberella zeae*; *L. maculans*, *Leptosphaeria maculans*; *M. oryzae*, *Magnaporthe oryzae*; *M. tuberculosis*, *Mycobacterium tuberculosis*; *N. crassa*, *Neurospora crassa*; *S. cerevisiae*, *Saccharomyces cerevisiae* and *Z. tritici*, *Zymoseptoria tritici*. (B) CLUSTAL Omega based amino acid sequence alignment of the conserved sites within the isocitrate lyase and methylisocitrate lyase proteins. Boxed amino acids show conserved amino acid change from phenylalanine (F) to leucine (L) and threonine (T) to serine (S) at substrate binding sites of the isocitrate lyase and methylisocitrate lyase proteins. Symbols (*) show identical amino acids, (:) conserved amino acids with strongly similar properties and (.) conserved amino acids with weakly similar properties.

A second BLASTP search of the *Z. tritici* genome database, using the *S. nodorum* MLS1 (NCBI accession XP_001797883) as a query, identified a single gene Mycgr3G70677 (Chromosome 4: 428,403-430,241) that encoded a 545 amino acid long peptide (NCBI accession XP_003853573). The Mycgr3G70677
peptide showed 83% identity to the *S. nodorum* MLS1. Protein domain analysis using ScanProsite (http://prosite.expasy.org/scanprosite/) identified a conserved malate synthase signature motif (RDHSSGLNCGRWDYIF) at amino acids 271 to 286 and a C-terminal tripeptide SKL motif (amino acids 543 to 545) required for peroxisomal targeting of the MLS1 proteins (Kunze et al., 2002). Based on phylogenetic and protein sequence analysis, the *Z. tritici* genes Mycgr3G102083, Mycgr3G83726 and Mycgr3G70677 were designated as the *ICL1*, *MCL1* and *MLS1* genes, respectively.

### 6.3.2 The *Z. tritici* ICL1, MCL1 and MLS1 genes complement loss of the glyoxylate and methylcitrate cycles in *S. cerevisiae*

To test whether the protein products of the *Z. tritici* genes *ICL1*, *MCL1* and *MLS1* were functional components of the glyoxylate and methylcitrate cycles, these *Z. tritici* genes were placed under the constitutive promoter $P_{GPD}$ and overexpressed in *S. cerevisiae*. The expression clones harbouring the promoter fusion to *Z. tritici* ICL1, MCL1 or MLS1 were introduced into the *S. cerevisiae* mutants Δicl1, Δicl2 and Δmls1 which individually lacked the *S. cerevisiae* genes *ICL1* (YER065C), *MLS1* (YNL117W) and *ICL2* (YPR006C), respectively. In *S. cerevisiae* ICL1 and MLS1 genes were indispensable for growth on ethanol as a sole carbon source (Kunze et al., 2002, Schöler and Schüller, 1993); and ICL2 was required for growth on odd chain carbon compounds such as glycerol (Luttik et al., 2000, Ferreira et al., 2005, Ronnow and Kielland-Brandt, 1993, Pavlik et al., 1993). The complemented strains Δicl1::ICL1 and Δmls1::MLS1 expressing the *Z. tritici* ICL1 and MLS1, respectively, grew comparably to the WT strain BY4741, irrespective of whether glucose or ethanol were present as the carbon source (Figure 6.4 A).
*cerevisiae* mutants Δicl1, Δmls1, and control strains Δicl1::P<sub>GPD</sub>:ccdB and Δmls1::P<sub>GPD</sub>:ccdB that carried the empty expression vector pAG426GPD-ccdB, failed to grow on ethanol as the sole carbon source. However, growth of these four mutants was comparable to that of the WT strain BY4741 on media containing glucose (Figure 6.4 A). On the media containing glycerol as a sole carbon source, growth of the strain Δicl2::MCL1 was comparable to that of the WT strain BY4741 (Figure 6.4 B). The mutant strains Δicl2 and Δicl2::P<sub>GPD</sub>:ccdB (strain Δicl2 carrying the empty expression vector) displayed a severely inhibited growth phenotype on media containing glycerol (Figure 6.4 B). As expected, the strains Δicl2, Δicl2::P<sub>GPD</sub>:ccdB, Δicl2::MCL1 showed normal growth both on glucose and ethanol (Figure 6.4 A). This demonstrated that the *Z. tritici* genes *ICL1*, *MLS1*, and *MCL1* functionally complemented the loss of the glyoxylate and methylcitrate cycles in *S. cerevisiae*. This suggests that products of these genes may have a similar role in *Z. tritici*. 
Figure 6.4 The *Z. tritici* ICL1, MCL1 and MLS1 genes functionally complement loss of orthologous *S. cerevisiae* genes.

The following *S. cerevisiae* strains were used; The gene deletion strains Δicl1, Δicl2 and Δmls1 lacking the *S. cerevisiae* ICL1, ICL2 and MLS1 gene individually; Complemented strains Δicl1::ICL1 (Δicl1::P_GPD:ICL1), Δicl2::MCL1 (Δicl2::P_GPD:MCL1) and Δmls1::MLS1 (Δmls1::P_GPD:MLS1) which constitutively expressed the *Z. tritici* gene ICL1, MCL1 or MLS1 under the control of *S. cerevisiae* P_GPD promoter; strains Δicl1::P_GPD:ccdB, Δicl2::P_GPD:ccdB and Δmls1::P_GPD:ccdB which carried the empty vector pAG426GPD-ccdB and the wild type strain BY4741. Cells were grown overnight in SC+2% (w/v) glucose at 30 °C and 180 rpm. Cells were washed with water and concentration adjusted to 10^7 cells mL^{-1}. A). Using a sterile loop, cells of *S. cerevisiae* each strain were streaked (as shown in Plate template) on SC agar containing 2% glucose or ethanol as sole carbon source. B). Serially diluted cells (10^5, 10^4, 10^3 and 10^2 cells mL^{-1}) of the strains Δicl2, Δicl2::MCL1, Δicl2::P_GPD:ccdB and BY4741 were spotted on SC agar containing 1% (v/v) glycerol as the sole carbon source.

6.3.3 Loss of the glyoxylate cycle affects spore development in *Z. tritici*

To determine the role of ICL1, MCL1 and MLS1 genes in *Z. tritici*, deletion mutants lacking these genes were constructed by introducing corresponding gene deletion vectors into the Δku70 strain HLS1000 via AMT (Figure 6.2 ABC). Diagnostic PCR analysis confirmed that the ICL1, MCL1 and MLS1 were replaced by the pILV2:ILV2^{SUR}, ptrpC:bar and ptrpC:php marker cassettes, respectively, in all the independent transformants of the mutant strains Δicl1, Δmcl1, Δmls1 and Δicl1Δmls1 (Figure 6.2 ABC). To represent each gene deletion strain, the independent *Z. tritici* transformants denoted as Δicl1.1, Δmcl1.1, Δmls1.1 and Δicl1Δmls1.1 were used for further analysis.
Figure 6.5 Analysis of lipid abundance, growth and enzyme activity in the *Z. tritici* strains lacking the ICL1, MCL1 and MLS1 genes.

Six-day old spores of the *Z. tritici* strains Δicl1.1, Δmcl1.1, Δmls1.1 and Δicl1Δmls1.1, and the background strain HLS1000 were used. (A) Spores treated with the lipophilic stains BODIPY (green fluorescence) and Nile red (red fluorescence) were exposed to the 488 nm solid-state laser for excitation and fluorescence was captured with the green channel detector (513-515 nm) and the red channel detector (560-565 nm) at 100x magnification using the Leica SP8 laser scanning confocal microscope. The bright-field and fluorescence channels were merged using Leica imaging suite. Bar = 10 µm. (B) Spores of each *Z. tritici* strain were inoculated on agarose cushion on a glass slide and micrographs were acquired at 0, 24 and 48 hours after inoculation. Micrographs were analysed using ImageJ to calculate spore-length; bars show mean (error bars show standard deviation) length calculated from 90 spores or hyphae per strain. Asterisks (*) indicate significant difference in mean spore length as compared to the strain HLS1000 at p ≤ 0.05. (C and D) Specific activities of the ICL (C) and the MLS (D) enzymes was analysed in the whole cell extracts prepared from strain after 24-hour growth in MM-Zt media containing 2% (w/v) sodium acetate or glucose as the sole carbon source. Bars show mean (error bars show standard deviation) of the specific enzymes activity (mU) per mg of total protein. Asterisks (*) show significant difference (p ≤ 0.05) from the strain HLS1000 in same condition. All experiments were repeated in biological triplicates. Statistical analysis conducted using analysis of variance (ANOVA).

To establish the role of ICL1, MCL1 and MLS1 genes during *in vitro* growth, spore-length and lipid abundance were analysed. Six-day-old spores of the mutant strains Δicl1.1, Δmcl1.1, Δmls1.1 and Δicl1Δmls1.1, and the background strain HLS1000 were stained with lipophilic stains BODIPY and Nile red, which bind to lipid bodies and emit green and red fluorescence, respectively (Rumin et al., 2015). Green and red fluorescence from individual and clumped subcellular organelles indicated that lipid-body abundance was qualitatively comparable between all four mutant strains and the background HLS1000 (Figure 6.5 A). Both fluorescence channels overlaid almost completely which confirmed that both BODIPY and Nile red bound to cellular lipids in a non-specific manner. These results indicated that none of the three genes detectably influenced lipid abundance in the *in vitro* grown spores of *Z. tritici*.
Additionally spore-length over time was measured to investigate whether inactivation of the \textit{ICL1}, \textit{MCL1} and \textit{MLS1} genes influenced \textit{in vitro} growth. To achieve this, spores of the mutant strains and the HLS1000 were inoculated on an agarose cushion on a glass slide and spore-length was measured at 0, 24 and 48 HAI (Figure 6.5 B). At 0 HAI, mean spore-length of the strains $\Delta mls1.1$ and $\Delta icl1\Delta mls1.1$ was reduced by one fourth as compared to that of the HLS1000. Conversely, spore-length was not affected in the strains $\Delta icl1.1$ and $\Delta mcl1.1$ and was comparable to that of the HLS1000 (Figure 6.5 B). Similar differences in spore or hyphal length between these strains persisted at 24 and 48 HAI. Overall, temporal reduction in spore or hyphal elongation suggested that \textit{in vitro} growth of the mutant strains $\Delta mls1.1$ and $\Delta icl1\Delta mls1$ was impaired, while no such defects were observed in the strains $\Delta icl1.1$ and $\Delta mcl1.1$ (Figure 6.5 B). These results showed that \textit{in vitro} growth of \textit{Z. tritici} was influenced by inactivation of the glyoxylate cycle and not the methylcitrate cycle. In addition results also revealed the inactivation of the \textit{MLS1} gene alone, or in combination with the \textit{ICL1} gene, caused significant growth defects as compared to none caused by inactivation of the \textit{ICL1} gene alone. Both the \textit{ICL1} and \textit{MLS1} genes encode for glyoxylate cycle enzymes and their dramatically different loss of function phenotypes were unexpected.

To test whether residual enzyme activity and phenotypic masking was the cause of the phenotypic variation resulted from the loss of \textit{ICL1} and \textit{MLS1} genes, we investigated the contribution of each gene to the corresponding enzyme activity. Whole cell extracts were prepared from the mutants and the background strain HLS1000 grown for 24 hours in media containing 2\% (w/v) glucose or sodium acetate as a sole carbon source. Extracts were analysed for \textit{ICL} and \textit{MLS} enzyme activities (Figure 6.5 C and D). Growth in sodium acetate
media increased the ICL activity of the strains Δ*mls1.1* and HLS1000 by 12 fold as compared to glucose media (Figure 6.5 C). ICL activities in the two mutants Δ*iicl1.1* and Δ*iicl1Δmls1* were severely reduced, and remained unaffected by either carbon source, and were similar to ICL activity of HLS1000 in glucose media. Similarly, MLS activities were 14 and 16 fold higher in the strains Δ*iicl1.1* and HLS1000, respectively, grown in acetate as compared to glucose media (Figure 6.5 D). Both the mutant strains Δ*mls1.1* and Δ*iicl1Δmls1* displayed severely reduced MLS activities that were independent of either carbon source, and were similar to the MLS activity of the strain HLS1000 in glucose media (Figure 6.5 D). Loss of ICL and/or MLS enzyme activities in the strains Δ*iicl1.1*, Δ*mls1.1* and Δ*iicl1Δmls1.1* confirmed that the *ICL1* and *MLS1* genes encoded for the corresponding glyoxylate cycle enzymes. We found that activities of both enzymes were elevated by acetate and repressed by glucose (Figure 6.5 C and D). Furthermore, glucose supressed ICL and MLS activities in the strain HLS1000 to the levels recorded for the mutant strain Δ*iicl1Δmls1.1*, which lacked the genes that encoded these enzymes. This suggested that the glyoxylate cycle was not required for growth by *Z. tritici* under glucose rich conditions. Together, these results suggest that the glyoxylate cycle is essential for normal growth and elongation of *Z. tritici* spores especially in glucose poor conditions and in presence of non-fermentable carbon sources such as acetate.

6.3.4 The glyoxylate cycle is required for full virulence of *Z. tritici*

To determine the role of *ICL1, MCL1* and *MLS1* genes in virulence of *Z. tritici*, a virulence assay was conducted on wheat seedlings (cultivar Riband). For this the mutant strains Δ*iicl1.1*, Δ*mcl1.1*, Δ*mls1.1* and Δ*iicl1Δmls1.1*, the background strain HLS1000 and the reference parental strain IPO323 (included
as an additional positive control) were inoculated on wheat leaves. Progress of disease symptoms was visually examined and disease severity was quantified by pycnidial density (pycnidia cm\(^{-2}\)) on the surface of infected leaves after 21 days (Figure 6.6). Disease progressed slowly in case of the mutant strains \(\Delta mls1.1\) and \(\Delta icl1\Delta mls1.1\); and chlorosis first appeared at 16 DAI followed by necrosis and sparse pycnidia becoming visible around 18 DAI (Figure 6.6). Both HLS1000 and IPO323, and the mutant strains \(\Delta icl1.1\) and \(\Delta mcl1.1\) induced typical STB infection symptoms (Figure 6.6) marked by emergence of chlorosis around 13 DAI; appearance of necrotic spots at 15 DAI and pycnidia were visible at 16-17 DAI. Mean pycnidial densities on the leaves inoculated with the strains \(\Delta mls1.1\) and \(\Delta icl1\Delta mls1.1\) were reduced by 60% and 85%, respectively, as compared to those induced by the background strain HLS1000 (Figure 6.6). Both the strain HLS1000 and IPO323 displayed relatively similar mean pycnidial densities. For the mutant strains \(\Delta icl1.1\) and \(\Delta mcl1.1\), mean pycnidial densities were mildly reduced as compared to HLS1000; however, reduction was statistically insignificant (Figure 6.6). These results illustrate that when the glyoxylate cycle is impeded/disrupted by inactivating the \(MLS1\) gene, virulence of \(Z.\ tritici\) on wheat leaves is severely reduced.
Figure 6.6 Virulence of the *Z. tritici* strains lacking the *ICL1, MCL1* and *MLS1* genes.

Spores of the mutant strains Δicl1.1, Δmcl1.1, Δmls1.1 and Δicl1Δmls1.1, the background strain HLS1000, and the reference wild type strain IPO323 were harvested following 6 day-growth on YG agar at 16 °C. Spore suspension (10^7 spores mL^-1) was inoculated on leaves of wheat seedlings (cultivar Riband) and after 21 days the infected leaves were imaged (as shown in the top panel). Pycnidia cm^-2 were counted manually and data for each strain was pooled and statistically analysed using analysis of variance (ANOVA). Bars show the mean pycnidia cm^-2 and error bars represent standard deviation for each strain. Asterisks (*) show significant difference (p ≤ 0.01) from the background strain HLS1000. The experiment was repeated in three biological replicates and each strain was inoculated in five technical replicates.

Hyphal growth is essential for *Z. tritici* to initiate disease (Mehrabi et al., 2006b). Therefore, it was reasoned that defects in spore elongation (Figure 6.5 B) could have an affect on hyphal growth, which may have caused reduced virulence in the mutants (Figure 6.6). To test whether this was the case, *in vitro* hyphal growth was initiated by growing the mutant strains Δmls1.1 and Δicl1Δmls1.1, and HLS1000 on wheat leaf agar media for 12 days at 25 °C. Wheat agar was used to mimic the nutritional environments faced by the pathogen during the course of infection. Fungal biomass was harvested and was stained with Nile red for microscopic examination. Both the mutant strains
Δmls1.1 and ΔiclΔmls1.1 produced short pseudohyphae, with extensively budding spores, as compared to the typical elongated and branched hyphae observed in case of the strain HLS1000 (Figure 6.7). Nile red staining of the strains Δmls1.1 and ΔiclΔmls1.1 showed strong red fluorescence signal, which indicated abundance of lipid bodies in the cytoplasm of the pseudohyphae and budding spores. In contrast, Nile red fluorescence intensity was greatly reduced in the elongated hyphae of the strain HLS1000 (Figure 6.7). Taken together, these results suggested that loss of the glyoxylate cycle affected virulence possibly by influencing lipid utilisation, which subsequently impaired infection related hyphal growth.
Figure 6.7 Analysis of hyphal growth and lipid abundance in the glyoxylate cycle mutant strains of *Z. tritici*.
Spores of the *Z. tritici* strains Δ*mls1.1* and Δ*icl1Δmls1.1*, and the background strains HLS1000 were inoculated on wheat leaf agar and incubated at 25 °C for 12 days. Fungal biomass was treated with Nile red (red fluorescence) and exposed to a 488 nm solid-state laser for excitation and fluorescence was captured with the red channel detector (560-565 nm) at 100x magnification using the Leica SP8 laser scanning confocal microscope. Micrographs acquired in the bright-field and fluorescence channel were merged using ImageJ. Scale bar = 10 µm.

6.3.5 Gluconeogenesis and glucose underpin *Z. tritici* infection

Results revealed that the activity of glyoxylate cycle enzymes were dispensable for growth of *Z. tritici* on glucose (Figure 6.5 C and D), however this pathway was required for full virulence of the fungus on wheat leaves (Figure 6.6). This led to speculation that *Z. tritici* could be utilising the glyoxylate cycle for glucose biosynthesis through gluconeogenesis and therefore exogenous glucose application could restore the virulence of the glyoxylate cycle mutants. To test this, a virulence assay was conducted, as described previously, using
the mutant strains Δmls1.1 and Δicl1Δmls1.1, and the background strain HLS1000. After every 48 hours, glucose or water solution was applied to the inoculated leaves. At 21 DAI, the leaves inoculated with the strains Δmls1.1 and Δicl1Δmls1.1, which had glucose application, displayed typical STB infection symptoms including chlorosis, necrosis and formation of pycnidia; and these symptoms were more severe compared to water application (Figure 6.8 A). The glucose application in case of the strains Δmls1.1 and Δicl1Δmls1.1 increased pycnidial densities by 4 and 4.5 fold, respectively, as compared to water application. With glucose application, the strains Δmls1.1 and Δicl1Δmls1.1 induced mean pycnidial densities comparable to those observed for the strain HLS1000 (Figure 6.8 B). For the strain Δicl1.1 mean pycnidial densities were relatively similar to the strain HLS1000, irrespective of glucose or water application. This demonstrated that exogenous glucose application indeed restored the virulence of the glyoxylate cycle mutants on wheat leaves.

To test if the increased virulence resulted from enhanced hyphal elongation, we analysed growth of the mutant strain Δicl1Δmls1.1 and the background strain HLS1000 on leaf surface at 12 DAI. Nile red was applied to leaf sections that were treated with glucose or water. The typical elongated and branched hyphae were ubiquitous on the leaves inoculated with the strain Δicl1Δmls1.1 and HLS1000 following glucose application (Figure 6.8 C). The Δicl1Δmls1.1 hyphae showed strong cytoplasmic Nile red fluorescence, which indicated cellular lipid abundance as compared to the hyphae of HLS1000. In the case of water application to inoculated leaves, spores were abundant on the surface of leaves inoculated with the strain Δicl1Δmls1.1 and these spores emitted a strong cellular Nile red fluorescence signal. The spores of the strain HLS1000 had differentiated into typical hyphae with relatively weak cytoplasmic
Nile red fluorescence signal and a bright signal from prominent enlarged subcellular organelles Figure 6.8 C. These results show that loss of the glyoxylate cycle affected lipid utilization and impaired the biosynthesis of glucose, which are required for normal hyphal growth and full virulence in *Z. tritici*. Taken together, results of this study demonstrate that the glyoxylate cycle plays a key role in the virulence of *Z. tritici* by linking lipid utilization, possibly through β-oxidation, to gluconeogenesis for production of glucose. This way the glyoxylate cycle serves to fuel the infection related morphological differentiation on the nutrient poor environments of the wheat leaf surfaces.
Figure 6.8 Application of glucose restored the virulence and hyphal growth of the glyoxylate cycle mutants.

Six-day-old spores of the mutant strains Δicl1.1, Δmls1.1 and Δicl1Δmls1.1, and the background strains HLS1000 were harvested from YG plates incubated at 16 °C. The second leaf of seventeen-day-old seedlings (cultivar Riband) was inoculated with spore suspension (10⁷ spores mL⁻¹). Using a paintbrush, one molar glucose or water solution was applied to infected leaves after every 48 hours for up to 21 days. Leaves were imaged after 21 days and are shown in the top panel (A). (B) Mean pycnidial densities (pycnidia cm⁻²) for each strain shown as bars; empty bars show 1 molar glucose treatment and grey filled bars for water treatment (error bars represent standard deviation). Pycnidia were counted manually and data was analysed using analysis of variance (ANOVA). Asterisks (*) show significant difference (p ≤ 0.01) from the background strain HLS1000 for each treatment. For each strain five technical replicates (leaves) were inoculated and experiments repeated in three biological replicate. (C) Glucose or water treated leaves inoculated with the mutant strain Δicl1Δmls1.1 and the background strains HLS1000 were detached after 12 days. Lipophilic stain Nile red (red fluorescence) was applied to small leaf sections and samples were exposed to a 488 nm solid-state laser for excitation and fluorescence was captured with red channel detector (560-565 nm) at 63x magnification using the Leica SP8 laser scanning confocal microscope. Micrographs acquired in the bright field and red fluorescence channel were merged using ImageJ. Bar = 25 μm.
6.4 Discussion

The glyoxylate cycle is an important metabolic pathway that facilitates utilisation of alternative carbon sources such as acetate in glucose deficient environments for net carbon assimilation by bypassing CO₂ producing steps of the TCA cycle (Figure 1 A) (Dunn et al., 2009). Various activators and repressors that function within the nutrient-sensing TOR (target of rapamycin) pathway mediate the transcriptional regulation of the glyoxylate cycle genes (Orzechowski Westholm et al., 2012, Vinekar et al., 2012). Glucose availability controls the regulatory mechanism known as carbon catabolite repression, which represses the glyoxylate cycle enzymes ICL and MLS under glucose rich conditions. In *S. cerevisiae*, glucose deficiency activates the SNF1 protein kinase that phosphorylates the RDS2 transcription factor (Ptacek et al., 2005), which binds in the promoters to activate the expression of the glyoxylate cycle genes *MDH1*, *MLS1* and *ICL1* (Soontorngun et al., 2007). In parallel, SNF1 also mediates phosphorylation of the glyoxylate cycle suppressor MIG1 (ortholog known as creA) leading to its disassociation from the *ICL1* and *MLS1* promoters. Therefore, phosphorylation of MIG1 is essential for the RDS2 mediated expression of the glyoxylate cycle genes. In addition, it has been speculated that the glyoxylate genes could also be in-directly regulated by the transcription factors *GIS1* and *RPH1* through down regulation of the transcriptional suppressor *XBP1* (Orzechowski Westholm et al., 2012).

In parallel, the glyoxylate cycle enzymes ICL and MLS are metabolically regulated by isocitrate diverted from TCA cycle. The phosphorylation of the TCA cycle component IDH1 (Isocitrate dehydrogenase) and subsequent suppression of IDH activity leads to diversion to isocitrate to ICL for conversion into
glyoxylate and subsequently into malate by MLS (Cozzone, 1998). Conversely, glucose abundance leads to dephosphorylation and elevation of IDH activity and consequently isocitrate is diverted back from the glyoxylate cycle into the TCA cycle (Cozzone, 1998, Gainey et al., 1992). The phosphorylation/dephosphorylation of IDH is controlled by the bi-functional isocitrate dehydrogenase kinase/phosphatase, which is widely conserved among prokaryotes and eukaryotes (Vinekar et al., 2012, Cozzone, 1998). Overall, the expression of the glyoxylate cycle genes is fine-tuned by the TOR mediated sensing of fermentable and non-fermentable carbon sources and transcriptional regulation of the carbon catabolic repression mechanism through SNF1, RDS2, MIG1 and the IDH kinase/phosphatase.

In the present study, we have shown that the glyoxylate cycle is required by *Z. tritici* to infect wheat and inactivation of this pathway influences growth by affecting gluconeogenesis mediated nutrient acquisition from stored lipids. Until now the mode and source of nutrition used by *Z. tritici* during initiation and the asymptomatic phase of STB infection cycle has been one of the most enigmatic aspects of the *Z. tritici*-Wheat pathosystem (Sanchez-Vallet et al., 2015). It was hypothesized that during the early stages of infection, *Z. tritici* acquired nutrition from the internally stored lipid and fatty acid reserves because the fungal mechanisms associated with transport and lipid metabolism were highly up regulated from the onset of infection (Rudd et al., 2015). Data from Rudd et al (2015) (Figure 6.9) revealed peak expression of the four genes namely *ICL1, MLS1, MDH1* (malate dehydrogenase) and *CIT2* (citrate synthase), which encode enzymes of the glyoxylate cycle, within 24 hours of infection. In addition, the expression of gene *ACS1* (acetyl-CoA synthase) and *GPR1* (glyoxylate pathway regulator), which are directly linked to the glyoxylate cycle...
(Dunn et al., 2009), was also highest within first 24 hours of infection. Similarly, expression pattern was observed in case of the genes CRC1 (carnitine transporter), FBP1 (Fructose-1,6-bisphosphatase; regulator of gluconeogenesis), SFC1 (Mitochondrial succinate-fumarate transporter), YAT1 and YAT2 (carnitine acetyltransferase), which encode for proteins involved in transport of the glyoxylate cycle substrates and by-products to peroxisomes, mitochondria and other organelles. These expression profiles of the genes involved directly or indirectly with the glyoxylate cycle were also observed in S. cerevisiae and C. albicans, which require the glyoxylate cycle for in vitro growth and virulence, respectively (Lorenz and Fink, 2001).

Figure 6.9 Expression profiles of the Z. tritici genes that encode the proteins associated with the glyoxylate cycle. Numbers represent mean FPKM values at each time point of infection. Gene expression data taken from Rudd et al., (2015).
Based on the phylogenetic analysis and amino acid conservation within the substrate binding sites, the *Z. tritici* ICL1 and MCL1 proteins coding genes were correctly resolved (Figure 6.3). The *MCL1* gene diverged from an ancient basidiomycete *ICL1* gene duplication event and due to high amino acid conservation with in the active sites, the *MCL1* genes are often incorrectly annotated as isocitrate lyase (Muller et al., 2011), which was the case with *Z. tritici*. Heterologous expression in *S. cerevisiae* confirmed that the *Z. tritici* ICL1, MCL1 and MLS1 proteins have same function in *S. cerevisiae* as illustrated by restored growth of the *S. cerevisiae* glyoxylate and methylcitrate cycle mutants on ethanol or glycerol, respectively (Figure 6.4). *S. cerevisiae* is an established model system for heterologous gene expression (Mokdad-Gargouri et al., 2012) and requires functional *ICL1*, *MLS1*, and *ICL2* genes for growth on non fermentable even and odd chain carbon sources, respectively (Kunze et al., 2002, Schöler and Schüller, 1993, Luttik et al., 2000, Ferreira et al., 2005, Ronnow and Kielland-Brandt, 1993, Pavlik et al., 1993). Similar to current study, heterologous complementation in *S. cerevisiae* has been exploited as a functional genomics tool to study emergence of drug resistance in *Z. tritici* through artificially induced target site mutations in the sterol 14α-demethylase encoding gene *CYP51* (Cools et al., 2011). *S. cerevisiae* was pivotal in elucidating the role of the β-1,3-glucan synthase gene *GLS1* from *C. graminicola* (Oliveira-Garcia and Deising, 2013). During the current study, complementation in *S. cerevisiae* provided the primary evidence that products of the *Z. tritici ICL1* and *MLS1*, the *MCL1* are involved in both the glyoxylate and methylcitrate cycles, respectively.
Primary microscopic analysis has revealed that the Nile red and BODIPY fluorescence were comparative between all the mutants and the WT strain. This implied that there were no obvious differences in the lipid content within the spores of the Z. tritici mutant lacking the ICL1, MCL1 and MLS1 genes and the WT strain (Figure 6.5 A). However, further quantification is required to test the hypothesis that deletion of these genes does not affect total cellular lipid content in Z. tritici. In model yeast species S. cerevisiae and S. pombe, the quantity and concentration of different types of cellular lipids is routinely quantified using a combination of lipophilic staining, fluorescence microscopy, and computational image analysis (Rostron et al., 2015, Sitepu et al., 2012). Although there are alternative lipid analysis techniques such as gravimetric analysis, and liquid and gas chromatography-mass spectrometry, however these are comparatively much more time consuming and require laborious lipid extraction and purification steps (Rostron et al., 2015, Borrull et al., 2015). Therefore, the fluorescence microscopy and image analysis is a simple and robust approach to reveal the influence of the glyoxylate or the methylcitrate cycles on cellular lipid content in Z. tritici.

Analysis of growth showed that the loss of the glyoxylate cycle through inactivation of the MLS1 gene alone and in combination with the ICL1, affected in vitro growth and temporal spore and hyphal elongation (Figure 6.4 B). However, these growth defects failed to manifest when the same pathway was inactivated through deletion of the ICL1 gene in the mutant strain Δicl1.1. This suggested that the contribution of the Z. tritici ICL1 and MLS1 to the glyoxylate cycle differed significantly and that the MLS1 has a more important role in this pathway. Similar to Z. tritici, the loss of MLS1 in S. nodorum inhibited spore germination and germ–tube elongation (Solomon et al., 2004). This points to a
a conserved role of the glyoxylate cycle in growth during nutrient poor conditions. In contrast to our results, deletion of the ICL1 gene in *M. oryzae* caused a delay in conidial germination and appresorium formation (Wang et al., 2003). Similar to *M. oryzae*, disruption of the ICL1 gene in *L. maculans* restricted elongation of the germinating spores (Idnurm and Howlett, 2002). However, as seen in *Z. tritici*, loss of both the ICL1 alleles of *C. albicans*, and loss of ICL1 and ICL2 (paralog of ICL1) in *M. tuberculosis* failed to affect in vitro growth (Lorenz and Fink, 2001, Munoz-Elias and McKinney, 2005). Thus, similar to *S. nodorum*, the glyoxylate cycle enzyme MLS is required for normal growth in *Z. tritici* while ICL is a dispensable component of this pathway. It is important to highlight that the spore and hyphal growth experiments that were conducted either on wheat leaf agar or agarose, and additional chain carbon compounds were not included. The role of glyoxylate and methylcitrate cycles in utilisation of odd and even chain carbon compounds dictates that follow up experiments must include quantification of growth on various fermentable and nonfermentable carbon sources. Such future experiment could be implemented through spotting assays as shown for *S. cerevisiae* in Figure 6.4, and subsequently colony diameter could be measured as an attribute of the radial growth phenotype.

In addition to affecting temporal spore and hyphal elongation, the loss of the glyoxylate cycle dramatically reduced virulence of the *Z. tritici* mutants on wheat leaves (Figure 6.5). To invade the leaf, *Z. tritici* spores need to develop into hyphae and inability to do so leads to attenuation or severely reduced virulence as was reported for the *Z. tritici* mutants that lacked the HOG1, FUS3, SLT2 or ALG2 gene (Mehrabi et al., 2006a, Mehrabi et al., 2006b, Cousin et al., 2006, Motteram et al., 2011) (Appendix 1). Thus, the virulence phenotype arose
from impaired infection specific hyphal growth displayed by the *Z. tritici* glyoxylate cycle mutants *in vitro* (Figure 6.6) and on wheat leaves (Figure 6.7 C). Similar to *Z. tritici*, the *S. nodorum* mutants lacking the *MLS1* gene showed a loss of pathogenicity due to restricted germ tube elongation which is required for stomatal penetration and for mechanical rupturing the leaf cuticle (Solomon et al., 2004). In contrast to the *MLS1*, the loss of *ICL1* and *MCL1* genes did not have a significant affect on pathogenicity of *Z. tritici* (Figure 6.5 A and B) or spore and hyphal elongation over time (Figure 6.4 B). This was not surprising because the *ICL1* and *MCL1* genes were dispensable for infection of wheat heads by *G. zeae* (Lee et al., 2009). As in *Z. tritici*, *ICL1* expression was highly up regulated during infection by the human pathogen *C. neoformans* however the *ICL1* deletion did not affect its virulence in mammalian infection models (Rude et al., 2002). Conversely, the loss of *ICL1* in *M. oryzae* reduced fungal virulence but mutants retained the ability to colonise rice leaves (Wang et al., 2003). In *L. maculans*, *ICL1* disruption severely reduced pathogenicity on oilseed rape cotyledons (Idnurm and Howlett, 2002). The most pronounced virulence related phenotype caused by the loss of *ICL1* was reported in the human pathogens *C. albicans* and *M. tuberculosis* as the mutants were rendered unable to survive within macrophages and were avirulent in mammalian infection models (Munoz-Elias and McKinney, 2005, Lorenz and Fink, 2001). Although, the *ICL1* gene plays a crucial virulence-associated role in a range of plant and human pathogens, we have illustrated that this was not the case for *Z. tritici*.

It is evident that the loss of ICL enzyme activity does not influence virulence in *Z. tritici* (Figure 6.6). Furthermore, the methylcitrate cycle is dispensable for *in vitro* growth, asexual reproduction and normal virulence of *Z.
tritici (Figure 6.5). The virulence phenotype was quantified based on a single attribute of infection, the pycnidial density that was recorded at 21 DAI. This time point was chosen because it is widely regarded to represent the peak of the symptomatic phase of Z. tritici infection during the in vitro virulence assays (Rudd et al., 2015, Motteram et al., 2009). The pycnidial density was a robust attribute in revealing the pronounced virulence defect in the ΔiclΔmls1.1 double mutant. However, it is possible that additional early-stage subtle virulence associated phenotypes were inadvertently missed due to the focus on the phenotype at 21 DAI. The STB infection is a dynamic process and it is therefore crucial to study multiple attributes of infection in order to establish a comprehensive link between mutations, perturbations and phenotypes. Therefore, it is important to reanalyze the virulence of the Δicl1.1 and Δmcl1.1 mutants by investigating multiple infection related attributes including the length of the asymptomatic phase, varying inoculum concentrations and virulence on wounded wheat leaves.

Reduced pathogenicity and growth defects in the Z. tritici mutants lacking a functional glyoxylate cycle were carbon source dependent (Figure 6.7 A, B and C) and restored by exogenous application of glucose. Similarly, glucose application also rescued full virulence of the S. nodorum mutant lacking MLS1 and the L. maculans mutants lacking ICL1 on wheat and oilseed rape, respectively (Solomon et al., 2004, Idnurm and Howlett, 2002). Thus glucose poor conditions on the leaf surface and inside apoplastic cavities of wheat leaves (Keon et al., 2007, Shetty et al., 2009) mean that the functional glyoxylate cycle is required for pre penetration growth of Z. tritici. Although, it has been illustrated that the glyoxylate cycle and gluconeogenesis mediated glucose biosynthesis is required for normal virulence, however, it is unknown
whether \textit{Z. tritici} can grow solely by utilising the sugars and host cytosolic components released following host cell death upon transition to the symptomatic phase (Shetty et al., 2009)? The glyoxylate cycle mutants failed to maintain normal growth on wheat leaf agar (Figure 6.7), which was anticipated to be abundant in host cytosolic contents. Thus, it can be hypothesised that host nutrients alone are not sufficient to initiate and sustain normal necrotrophic growth of \textit{Z. tritici}. To test this hypothesis, follow up experiments must be conducted which include application of glucose solution on to the infected leaves up to the time point of transition to the symptomatic phase of infection. The capability of \textit{Z. tritici} to survive solely on host nutrients would confirm that lipid metabolism provides energy for the minimal fungal growth during the asymptomatic phase and abundance of host-nutrients from the onset of symptomatic phase fuels the dramatic increase in the necrotrophic fungal growth.

The \textit{Z. tritici} ICL and MLS enzyme activities were supressed to background levels during growth on glucose while these enzymes were highly induced in presence of acetate (Figure 6.5 C and D). Acetate skeletons and Acetyl-CoA originating from lipid degradation were proposed as the key carbon source during early stage of \textit{Z. tritici} infection (Rudd et al., 2015). Indeed, we saw that inactivation of the glyoxylate cycle may have influenced lipid utilization as apparent from the increased Nile red fluorescence signal originating from a large number of lipid bodies present within the spores and pseudohyphae of the glyoxylate mutant strains \textit{in vitro} (Figure 6.7) and \textit{in planta} (Figure 6.8 C). Lipid droplets are transported into the vacuoles where, upon autophagy these are degraded and this coincides with vacuole enlargement (Weber et al., 2001, Singh et al., 2009). Reduced cytoplasmic fluorescence and strong fluorescence...
signal from the enlarged vacuoles of within the hyphae of the strain HLS1000 (Figure 6.7 and Figure 6.8 C) provided evidence to support normal lipolysis, which was absent in the glyoxylate cycle mutants. Similarly, lipid mobilization and subsequent lipolysis was inhibited in the S. nodorum and M. oryzae mutants lacking the MLS1 and ICL1 genes, respectively (Wang et al., 2003, Solomon et al., 2004). This implied that as seen in M. oryzae and S. nodorum (Wang et al., 2003, Solomon et al., 2004) inactivation of the glyoxylate cycle in Z. tritici could have influenced lipid transportation, which in turn affected the β-oxidation of lipids within peroxisomes. Impaired cellular lipid trafficking raises the question whether loss of the glyoxylate cycle affected activity of cytoplasmic lipases that carry fatty acids between lipid droplets and sites of lipolysis, the mitochondria and peroxisomes (Rambold et al., 2015). Further investigation will be required to understand how loss of the glyoxylate cycle influences lipolysis. Based on our results, it has emerged that infection specific growth in Z. tritici is likely to be fuelled by lipid catabolism and subsequent channelling of the by-products, via the glyoxylate cycle, into gluconeogenesis for glucose biosynthesis.

The dispensability of ICL enzyme for growth of Z. tritici in the glucose rich conditions and its requirement for utilisation of acetate as a carbon source is consistent with the observation of Rohel et al., (2001). These authors monitored regulation of the Z. tritici ICL1 over the course of STB infection by expressing a transcriptional fusion of the N. crassa acu-3 (ICL1 ortholog) promoter and the GFP reporter. The measurement of GFP fluorescence and western blots revealed up regulation of GFP protein expression at pre penetration stages and expression subsequently declined throughout the course of STB infection. Based on these observations, Rohel et al., (2001) proposed that ample glucose
and sugar content within the host apoplastic fluid meant that *Z. tritici* ICL1 was likely to be dispensable during post penetration stages of infection. This speculation has been reinforced by dispensability of the *Z. tritici* ICL1 (Figure 6.5, 6.6, 6.7 and 6.8). However, these results raised the question that if apoplastic glucose content was sufficient for the infectious growth of *Z. tritici*, then why did the other two mutants Δmls1.1 and Δicl1Δmls1.1, which also lack the glyoxylate cycle, failed to maintain normal growth and virulence phenotypes?

It could be possible that the loss of ICL activity in the *Z. tritici* Δicl1.1 mutant was functionally complemented by MCL as both the ICL1 and MCL1 proteins share high amino acid conservation within the active substrate binding sites (Figure 6.3). In the wheat pathogen *G. zeae*, loss of the ICL1 gene lead to elevated expression of the MCL1 gene and this was speculated to be a compensatory mechanism to complement the loss of *G. zeae* ICL enzyme activity (Lee et al., 2009). Furthermore, functional complementation between the glyoxylate and the methylcitrate cycle enzymes has also been reported in *M. tuberculosis* (Gould et al., 2006). Therefore, functional complementation of the ICL by MCL enzyme is a plausible explanation. However, the enzyme assay on the Δicl11.1 mutant (Figure 6.5 C and D) showed that the *Z. tritici* MCL failed to reduced isocitrate into succinate and glyoxylate, as would be anticipated in case of a functional ICL enzyme (Figure 6.1 A, Figure 6.5). Therefore, it is evident that *Z. tritici* MCL is unlikely to complement for the loss of ICL. Therefore, future investigations are crucial to elucidate the mechanisms that compensate for the loss of ICL activity. The in depth phenotypic analysis of the double mutant that lacks both the *Z. tritici* MCL1 and ICL1 genes would provide
crucial data required to confirm or eliminate the redundancy between ICL and MCL enzymes.

The results have illustrated that although the ICL mediated glyoxylate biosynthesis is dispensable, however, glyoxylate is required for production of malate by MLS for normal growth and virulence of *Z. tritici* in glucose limiting conditions (Figure 6.5 B and Figure 6.6). In absence of ICL, such a scenario could only be possible if sufficient cellular glyoxylate is available to MLS for conversion into malate. Thus, it is reasonable to speculate that a modified form of the glyoxylate cycle may be functional in *Z. tritici*. To confirm the existence of this hypothetical ICL bypass mechanism, it is essential to elucidate the source of cellular glyoxylate metabolite. It could be hypothesized that *Z. tritici* may have evolved a novel mechanism similar to that reported in the cultivated potato (*Solanum tuberosum*) whereby glyoxylate is synthesized by condensing two molecules of formate (Ramaswamy et al., 1983, Janave et al., 1993, Bar-Even et al., 2011). This is reasonable because formate is a cellular metabolite that is abundantly synthesised by pathogenic fungi (Jefferson and Foster, 1953, Müller et al., 2012, Kuwazaki et al., 2003). However, our in-depth search of the *Z. tritici* genome failed to identify an ortholog of the glyoxylate synthetase gene from potato (Ramaswamy et al., 1983). Therefore, additional genomic and metabolic analyses are required to conclusively establish whether such a glyoxylate synthetase gene is likely to exist in *Z. tritici*. Alternatively, glyoxylate could be produced from reduction of complex fungal cellular metabolites such as methylglyoxylate, phenylglyoxylate, and methyl phenylglyoxylate by demethylation and/or dephenylation (Duan et al., 2014, Wang et al., 2006). Undoubtedly, future investigations involving metabolic profiling for cellular glyoxylate content and in-depth phenotypic screening of *Z. tritici* double mutant
lacking both *ICL1* and *MCL1* would serve to address the ambiguity regarding the role of glyoxylate cycle enzymes in *Z. tritici*.

Consistent with *Z. tritici*, *ICL1* orthologs were highly unregulated at various stages of infection by human pathogens *A. fumigatus* and *C. neoformans*, although ICL enzymes were dispensable for normal virulence of both these pathogens (Schobel et al., 2007, Rude et al., 2002). It was speculated that ICL was dispensable either due to abundance of glucose within the mammalian hosts or loss of the glyoxylate cycle was complemented by the methylcitrate cycle as an alternative carbon metabolic pathway (Schobel et al., 2007, Rude et al., 2002). Indeed the follow up investigations have confirmed that the methylcitrate cycle is functional carbon metabolic pathway, which provides energy to sustain normal growth and virulence of *A. fumigatus* (Ibrahim-Granet et al., 2008). However, to date it remains unclear whether such an alternative carbon metabolism pathway is also functional in *C. neoformans*. Consistent with Schobel et al., (2007) and Rude et al., (2002) it could be speculated that abundance of host glucose means that invading pathogens do not face nutritional starvation. However, if host glucose content was sufficient, then why do several pathogens including *S. nodorum*, *C. albicans*, *M. tuberculosis*, *M. oryzae* and *Z. tritici* require functional glyoxylate cycle and gluconeogenesis to complete their life cycles? The requirement of glyoxylate cycle and gluconeogenesis for fungal virulence in a range of mammalian and plant pathogens illustrates that majority of these pathogens face nutritional deficiency during pre penetration stages of infection (Lorenz and Fink, 2001, Thines et al., 2000). During such nutritional deficiency periods, the glyoxylate and/or the methylcitrate cycle serve to utilise stored energy reserves and to overcome starvation. Overall, it is evident that future investigations are crucial to
elucidate the role of individual glyoxylate cycle enzymes in nutrient acquisition by plant and mammalian pathogens. Based on results of the current study, it is also clear that the glyoxylate cycle must be completely inactivated through double deletion of the *ICL1* and *MLS1* in order to conduct a comprehensive characterisation of this metabolic pathway.

Due to indispensability of the glyoxylate cycle for virulence, the *Z. tritici* ICL and MLS enzymes could have served as ideal drug targets. However, wheat genome sequence (Brenchley et al., 2012) also contains the *ICL1* and *MLS1* orthologs that encode for proteins with malate synthase (UniProt ID - W5BR01) and isocitrate lyase activities (UniProt ID - D2KZ15). The wheat malate synthase protein is 38.3% identical and 48.6% similar to the *Z. tritici* MLS1, while the wheat isocitrate lyase shows 52 % identical and 66.4 % similar to the *Z. tritici* ICL1 protein. Such high amino acid identifies and conserved active substrate binding sites imply that any drug molecule designed to target the *Z. tritici* ICL1 and MLS1, is also likely to target the orthologous proteins in wheat. As a result the *Z. tritici* ICL1 and MLS1 could not be exploited as drug targets for development of new fungicides. However, the networks that regulate the glyoxylate cycle enzymes and proteins involved in essential substrate trafficking, to and from the glyoxylate cycle (Figure 6.9), may yield novel drug targets for early intervention fungicides.

Overall the current study has revealed that the glyoxylate cycle is required by *Z. tritici* for initiation of STB infection. Consistent with its role in other pathogenic fungi, it is possible that this pathway enables *Z. tritici* to utilise the by products of stored lipid metabolism to fuel the hyphal growth. The findings of this study provide a conclusive evidence to speculate that *Z. tritici* is likely to
rely solely on stored lipid reserves for hyphal growth during pre penetration and the asymptomatic phase of infection. Consequently, these results reinforce that *Z. tritici* is not a hemibiotrophic but is a necrotrophic fungus with an extended asymptomatic or latent phase of infection (Rudd, 2015).

### 6.5 Conclusions

We have answered a key question of fungal nutrition during early stages of STB infection by showing that the glyoxylate cycle and lipid catabolism underpin the full virulence of *Z. tritici* on wheat. This observation strengthens the existing role of the glyoxylate cycle as a virulence factor in pathogenic bacteria and fungi. This study also showed that the methylcitrate cycle was dispensable during infection by *Z. tritici*. We have shown that in the absence of fermentable carbon sources, *Z. tritici* engages the glyoxylate cycle to produce complex carbohydrates required to fuel hyphal growth for host colonization. Due to its role in fungal virulence, the druggable components within the transcriptional networks that regulate the glyoxylate cycle could be exploited as targets to develop new fungicides.
Chapter 7

General discussion and future research directions
7.1 Importance of functional genomics in *Z. tritici*

*Z. tritici* is a major threat to global wheat production (Goodwin et al., 2011), however, despite the economic importance and its genetic tractability, *Z. tritici* is a relatively understudied pathogen, as compared to other plant pathogenic fungi (Dean et al., 2012, Perez-Nadales et al., 2014). This has led to gaps in the knowledge regarding the biology of fundamental virulence associated processes such as the state of dispersal, adherence, germination and nutrition mechanisms of the infectious propagules to evasion and subversion of host defences (Chapter 1, section 1.3). Majority of *Z. tritici* research focuses on identification of the virulence associated effectors (Mirzadi Gohari et al., 2015, Rudd et al., 2015) which are key to understand the Wheat-*Z. tritici* interactions and identification novel sources of genetic resistance (Vleeshouwers and Oliver, 2014). However, it is equally important to investigate the biological processes that underpin virulence of *Z. tritici* through germination, morphogenesis and stress tolerance during host colonisation.

Modern comparative genomics tools can be utilised to identify the proteins involved in ubiquitous virulence-related biological processes. However, due to evolutionary rewiring of biological networks, the specific functions of a predicted protein can vary significantly between two organisms. For example, the downstream targets regulated by the transcription factor CRZ1 (Calcineurin Responsive Zinc finger 1), which regulates calcium mediated signalling, differed significantly between *M. oryzae*, *S. cerevisiae* and *A. fumigatus* (Kim et al., 2010). Similarly, functional differences between orthologous proteins complicated effector characterisation as was demonstrated by dispensability of the *Z. tritici* MgNLP (Motteram et al., 2009) which is an ortholog of the effector
proteins required for full virulence of *Phytophthora capcisi* on pepper (Feng et al., 2014) and *Erwinia carotovora* subsp. *carotovora* on potato (Mattinen et al., 2004). Furthermore, due to polymorphism and lack of homology to known proteins, the roles of rapidly evolving and positively selected *Z. tritici* specific proteins such as Zt80707 and Zt103264, which influence virulence of this pathogen (Poppe et al., 2015), cannot be inferred through homology based sequence analysis. Therefore, the roles of proteins encoded by the candidate *Z. tritici* genes need to be functionally characterised to address the issues arising from functional variation and redundancy.

Functional genomics require a variety of molecular tools to modulate and manipulate cellular genetic and metabolic content in order to elucidate protein function, complex transcriptional regulatory networks and identify novel drug targets. Until recently, the molecular tools required for medium to high-throughput functional genomics in *Z. tritici* were unavailable. Lack of such tools and consequently the slow pace of functional analyses in *Z. tritici* implies that it would take more than half a century to characterise 1% of the *Z. tritici* genome (Cairns et al., 2015). To address this bottleneck, the current study was conducted with an aim to develop molecular tools that would facilitate large-scale functional genomics analyses in *Z. tritici*.

The main objectives of the current study were 1) to establish a reproducible genetic transformation protocol; 2) to construct the *Z. tritici* strains that facilitate high frequency genome editing; 3) to establish gene overexpression as mode of functional analysis; and 4) to establish a strategy for regulatable gene expression and verification of gene essentiality in *Z. tritici*. These objectives were successfully accomplished and the tools have been
developed and disseminated to the *Z. tritici* research community (Sidhu et al., 2015a, Sidhu et al., 2015b, Marchegiani et al., 2015). In addition, these molecular tools enabled Cairns et al., (2015) to establish a platform for high-throughput phenotypic screening of *Z. tritici* overexpression strains (Cairns et al., 2015). Furthermore, by illustrating the role of glyoxylate cycle and lipolysis during pre-penetration morphogenesis of *Z. tritici* (Chapter 6), these molecular tools served to reveal novel biology and answer fundamental questions regarding source and mechanism of fungal nutrition during the asymptomatic phase of STB infection.

### 7.2 New opportunities for forward and reverse genetics in *Z. tritici*

The lack of high-throughput vector construction techniques, reproducible genetic transformation methods, positive selection markers and low frequency of targeted genome manipulation are primary hurdles in functional genomics through systematic large-scale gene deletion projects (Collopy et al., 2010, Schuster et al., 2012). In the current study, the yeast recombinational cloning compatible ternary vectors were developed to facilitate rapid vector construction for AMT of *Z. tritici* (Sidhu et al., 2015a)(Chapter 3). In addition to plasmid construction for current research, the yeast recombinational cloning has been pivotal in high-throughput plasmid construction for advanced cell biology of *Z. tritici* (Kilaru and Steinberg, 2015, Kilaru et al., 2015b) and large-scale gene deletion projects in *Trichoderma reesei* (Schuster et al., 2012) and *N. crassa* (Colot et al., 2006, Collopy et al., 2010, Park et al., 2011a). Therefore, the yeast recombinational cloning compatible ternary vectors will facilitate plasmid construction for molecular and cell biology of in *Z. tritici* and other organisms amenable to AMT.
The high-throughput genetic manipulation techniques were pivotal in large-scale functional genomics analysis in *S. cerevisiae* (Giaever et al., 2002, Giaever and Nislow, 2014), *C. glabrata* (Schwarzmuller et al., 2014), *N. crassa* (Park et al., 2011a, Collopy et al., 2010) and *M. oryzae* (Wilson and Talbot, 2009). The irreproducibility of the protoplast transformation methods reported for *Z. tritici* (Skinner et al., 1998, Payne et al., 1998, Rohel et al., 2001, Adachi et al., 2002) meant that it was not possible to establish such a high-throughput transformation method for *Z. tritici*. Therefore, the AMT of *Z. tritici* was optimised and the resulting highly reproducible protocol (Chapter 2 section 2.4)(Sidhu et al., 2015a) offers a higher throughput as compared to the protocol by Zwiers and De Waard (2001). As a result of increased throughput, Cairns et al., (2015) introduced the thirty-two *Z. tritici* gene overexpression vectors in a single transformation experiment. Therefore, elevated reproducibility and throughput makes the optimised AMT protocol an ideal tool for construction of libraries of *Z. tritici* insertional mutants strains that can be used for forward genetic screens. For instance genome sequencing of the viable insertional mutants will enable identification of the dispensable *Z. tritici* genes that can be disrupted and this dataset could be used to identify the putative essential genes, which are indispensable and hence ideal drug targets. In parallel, the *Z. tritici* insertional mutants can be screened for virulence defects using the high-throughput infection assay (Torriani et al., 2015). Similarly, mutants can be screened in presence of infection related stress conditions such as oxidative, osmotic and antifungal agents by using high-throughput *in vitro* phenotyping platform (Cairns et al., 2015). In *M. oryzae*, a similar forward genetic screen revealed more than two hundred loci that regulate various virulence-associated functions including conidial growth, morphogenesis and appressorium formation, along with
identification of novel drug targets such as the *M. oryzae* chitin synthase activator 3 gene (MGG_03530) (Jeon et al., 2007). This example from *M. oryzae* highlights that forward genetic screens have a huge potential to facilitate identification of novel biological mechanisms that regulate virulence of *Z. tritici* in wheat.

The low frequency of targeted genome manipulation in *Z. tritici* has withheld application of reverse genetic screens through loss of function analysis and gene deletion. This bottleneck has been addressed by constructing the *Z. tritici* mutant strains Δku70 and Δku80, which lack the *KU70* or *KU80* genes, respectively, (Sidhu et al., 2015a)(Chapter 4) and enable high frequency targeting of various *Z. tritici* genes and overexpression cassettes (Chapter 3, 4, 5 and 6)(Cairns et al., 2015). In addition, the Δku70 strain facilitated construction the *Z. tritici* double mutant strain Δicl1mls1.1, which served to address the disparity between the loss of glyoxylate cycle phenotypes that arose from individual inactivation of the *Z. tritici MLS1* or *ICL1* gene (Chapter 6, section 6.5). As a result, these molecular tools illustrated that the glyoxylate cycle enables *Z. tritici* to channel the by-products of lipolysis into gluconeogenesis to fuel pre-penetration morphogenesis required for initiation of STB disease cycle.

This link between the glyoxylate cycle and virulence of *Z. tritici* has shed new light on the trophic nature and the source of nutrition during early stages of STB infection, whilst opening new opportunities for fungicide discovery. For instance, the druggable components of the lipids biosynthesis and substrate transport pathways (Chapter 6, section 6.4) could be exploited as potential drug targets. In addition, for example, in *M. oryzae* the MAPK PMK1 mediates
trafficking of cytosolic lipids to vacuoles for lipolysis (Wang et al., 2007, Thines et al., 2000) and the transcription factor STE12, which is activated by the PMK1 ortholog FUS3, regulates lipid utilization in *C. lagenarium* (Tsuji et al., 2003). Therefore, the mechanisms involved lipid transport and utilisation in *Z. tritici* could be explored by examining the *Z. tritici* mutant strains Δfus3 and Δste12, which lack the *PMK1* ortholog *Z. tritici FUS3* and the *STE12* genes, respectively, and showed severe virulence defects on wheat leaves (Cousin et al., 2006, Kramer et al., 2009). If lipid mobilisation is affected in the *Z. tritici* Δfus3 and Δste12 mutants, subsequent analysis of the regulatory targets of STE12 through chromatin immunoprecipitation-sequencing (ChIP-seq) could help to uncover druggable component within the lipid biosynthesis and trafficking pathway. A similar analysis of transcriptional networks that regulate the *Z. tritici CBR1* and *CBR2* genes, which encode cytochrome b5 reductase proteins that influence lipid biosynthesis and virulence in various fungal pathogens (Derbyshire et al., 2015, Nguyen et al., 2012, Zhao et al., 1996) could also reveal potential fungicide targets in *Z. tritici*.

The ability to inactivate multiple genes in the same genome background will be especially useful in addressing the functional redundancy, which presents a major challenge in characterisation of the *Z. tritici* effectors (McDonald et al., 2015, Rudd et al., 2015, Mirzadi Gohari et al., 2015). Similarly, multiple-gene deletion mutants are crucial to completely inactivate the pathways that involve activity of multiple proteins encoded by paralogous genes (Hentges et al., 2005). For example, in *A. fumigatus* it was essential to inactivate the three paralogous α-(1,3)-glucan synthase genes to completely eliminate the cell wall α-(1,3)-glucan content (Henry et al., 2012) in order to reveal that this polysaccharide fraction is required for PTI evasion and infection.
related morphogenesis during invasive pulmonary aspergillosis (Beauvais et al., 2013). Thus, ability to inactivate the three paralogous Z. tritici α-(1,3)-glucan synthase (AGS) genes could serve to elucidate whether this cell wall polysaccharide plays a protective role in Z. tritici, as it does in case of M. oryzae and A. fumigatus (Fujikawa et al., 2012, Beauvais et al., 2013). Out of the three Z. tritici AGS genes, two namely AGS1 (Mycgr3G72646) and AGS2 (Mycgr3G99568) show elevated expression during the asymptomatic phase (at 4 DAI) and at the onset of the symptomatic phase (at 9 DAI), respectively, while the third gene AGS3 (Mycgr3G32133) is relatively silent throughout the STB infection cycle (Figure 7.1). This stage specific expression of the Z. tritici AGS1 and AGS2 suggests that these proteins may serve to prolong the asymptomatic phase by delaying detection and protecting the fungus against fungal cell wall lysing enzymes produced by the host. If the Z. tritici AGS proteins are found to influence fungal virulence, then the components of the α-(1,3)-glucan biosynthesis pathway in this fungus could be exploited to discover novel active ingredients to develop fungicides for early stage disease intervention strategies.

![Figure 7.1 Expression profiles of the Z. tritici α-(1,3)-glucan synthase genes. Data taken from Rudd et al., (2015).](image)

Figure 7.1 Expression profiles of the Z. tritici α-(1,3)-glucan synthase genes. Data taken from Rudd et al., (2015).
In addition to lack of functional analysis through gene inactivation, current study also reports use of the nitrogen responsive promoters, $pMoNIA1$ and $pZtNIA1$ from $M. oryzae$ and $Z. tritici$, respectively, to provide tight up and down regulation of gene expression in $Z. tritici$ (Chapter 5)(Marchegiani et al., 2015). Ability to modulate gene expression is a prerequisite for studying essential genes (Chauvel et al., 2012, Ouyang et al., 2015). Indeed the $pZtNIA1$ promoter facilitated knock-down of the $Z. tritici BGS1$ gene expression, which encodes a $\beta$-(1,3)-glucan synthase which plays a major role in cell wall stress tolerance and is likely to be essential; hence a potential drug target (Chapter 5). Thus, the regulatable promoters have opened new opportunities for systematic characterisation of the essential genes in $Z. tritici$ (Kilaru et al., 2015a, Marchegiani et al., 2015). In parallel, controlled gene expression will serve to study function of the proteins that are lethal upon constitutive expression. For example, due lethality of the proteins in $S. cerevisiae$, Cools et al., (2011) were unable to express the lethal mutant alleles of the $Z. tritici CYP51$ gene in order to interrogate the role of potential target site mutations in azole fungicide resistance mechanisms (Cools et al., 2011). In future, such studies could be conducted in $Z. tritici$, as opposed to heterologous expression in $S. cerevisiae$, by placing the lethal protein coding genes under the control of the $pZtNIA1$ promoter and on-demand gene expression and repression can be achieved in presence of inducing and repressing conditions (Chapter 5).

Overall the $Z. tritici \Delta ku70$ and $\Delta ku80$ mutant strains, the optimized AMT protocol including the yeast recombinational cloning and the new positive selection marker $pILV2:ILV2^{SUR}$, and the regulatable promoters would facilitate large-scale reverse genetics screens in $Z. tritici$. The resulting data will fill in the
current gaps in knowledge of the infection related biology whilst also revealing new drug targets and drug resistance mechanisms in Z. tritici.

7.3 Future directions for gene overexpression in Z. tritici

Until recently, gene overexpression and proteins tagging was rarely used for functional genomics in Z. tritici. This was addressed by developing a versatile suite of 32 expression vectors that allow flexible cloning of ORFs for gene overexpression and protein tagging in this fungus (Sidhu et al., 2015b)(Chapter 4). Using these vectors, Cairns et al., (2015) conducted first large-scale gene overexpression study involving overexpression of thirty-two Z. tritici genes that encode the transcription factors and kinases. As a result, Cairns et al., (2015) revealed that constitutive overexpression of the transcription factor-coding gene AlmA (Mycgr3g111569) impeded hyphal growth (Cairns et al., 2015). The hyphal growth is essential STB disease initiation (Mehrabi et al., 2006b) therefore, the screen by Cairns et al., (2015) illustrates that gene overexpression and subsequent gain of function analysis will open new opportunities for elucidation of complex pathways that underpin virulence of Z. tritici. Similarly, large-scale gene overexpression screen in the human pathogen C. albicans identified several genes that regulate hyphal morphogenesis required for initiation of invasive candidiasis infections (Chauvel et al., 2012). Likewise, overexpression of S. cerevisiae genes revealed new drug targets, novel signalling and gene regulatory pathways through analysis of protein kinases and transcription factors, and the resulting knowledge has furthered the understanding of eukaryotic biology (Stevenson et al., 2001, Chua et al., 2006, Prelich, 2012, Gelperin et al., 2005).
The gene overexpression and gain of function analysis will serve to circumvent functional redundancy, which is a major bottleneck in functional genomics through loss of function analysis via targeted gene deletion (Prelich, 2012). Therefore, gene overexpression will be particularly useful in characterisation of the putative effector coding genes in *Z. tritici*. For instance, overexpression the nineteen *Z. tritici* genes, which encode secreted chloroperoxidase proteins (Rudd et al., 2015), could reveal whether these proteins enable *Z. tritici* to sequester ROS that are produced by the host during various stages of STB infection cycle (Shetty et al., 2007). In parallel, overexpression of the forty-two *Z. tritici* genes that encode ROS sequestering peroxidases and catalases (Mir et al., 2015) could serve to elucidate the biological mechanisms that enable *Z. tritici* to grow in presence of host immune response. For instance, an ideal starting point will be to overexpress the three *Z. tritici* genes Mycgr3G85387, Mycgr3G67250 and Mycgr3G102589, which are orthologous to the *M. oryzae* genes *CATB*, *CXPB* and *HYR1* that encode secreted large subunit catalase, catalase peroxidase and glutathione peroxidase, respectively, and are required for oxidative stress resistance and/or full virulence of *M. oryzae* on rice leaves (Skamnioti et al., 2007, Tanabe et al., 2011, Fernandez and Wilson, 2014, Mir et al., 2015). Such studies are essential to uncover the role of oxidative stress resistance mechanisms in *Z. tritici* during the asymptomatic phase of STB infection.
Figure 7.2 Expression profiles of the selected *Z. tritici* peroxidase coding genes. Data taken from Rudd et al., (2015).

The high-throughput *in vitro* phenotypic screening platforms (Torriani et al., 2015, Cairns et al., 2015) coupled with gene overexpression could reveal the roles of *Z. tritici* ABC transporters, which are expressed at various stages of STB infection (Rudd et al., 2015, Kellner et al., 2014). ABC transporters modulate fungal virulence through pleotropic drug efflux and stress resistance, and by sub-cellular trafficking of essential substrates (Kovalchuk and Driessen, 2010, Roohparvar et al., 2007, Zwiers et al., 2007, Roohparvar et al., 2008, Stergiopoulos et al., 2002, Stergiopoulos et al., 2003, Sun et al., 2006). In addition, more than half of the forty-eight human ABC transporters are involved in lipid trafficking (Tarling et al., 2013) and requirement of lipid mobilisation and utilisation for virulence of *Z. tritici* (Chapter 6) dictates that the ABC transporters are likely to influence virulence of this fungus. The virulence-associated role of the *Z. tritici* ABC transporters is further supported by their proposed function in transport of host metabolites and antifungal compounds produced during
compatible interactions (Yang et al., 2013b, Brunner et al., 2013, Yang et al., 2013a). Out of the forty-six ABC transporters encoded by the Z. tritici genome, only six have been functionally characterised (Appendix 1) and five are dispensable for STB infection, although two of these Z. tritici ATR1 and ATR7 regulate azole resistance and iron homeostasis, respectively (Zwiers et al., 2002, Zwiers et al., 2007, Stergiopoulos et al., 2003). Only one ABC transporter the ATR4 is required for full virulence and inactivation affects colonisation of sub-stomatal cavities by Z. tritici (Stergiopoulos et al., 2003). Therefore, systematic overexpression of all the Z. tritici ABC transporters would serve to uncover their role in stress tolerance and antifungal resistance during STB disease cycle.

In addition to gene overexpression, these expression vectors allow protein tagging in Z. tritici, which opens new opportunities for cell biological investigations of the Wheat- Z. tritici interactions (Sidhu et al., 2015b). One potential application of cell biology will be to investigate the role of secreted Z. tritici effectors in modulation of host defence responses during STB infection (Rudd, 2015). Equally, cell biology could be applied to interrogate the biology of morphogenesis and polarised hyphal growth by studying the cytoskeletal components such as the guanosine-5'-triphosphate (GTP) binding septin proteins, which regulate cytokinesis, polarised cell growth and effector secretion in fungi (Berepiki and Read, 2013, Dagdas et al., 2012, Gupta et al., 2015). An ideal starting point for such investigation will be to study spatial and temporal subcellular protein localisation by tagging the Z. tritici genes CDC3 (Mycgr3G62823) and CDC12 (Mycgr3G100895) genes, orthologs of which encode proteins that negatively regulate cell polarity to limit sites of hyphae or germ tubes emergence in N. crassa and C. albicans (Berepiki and Read, 2013,
Li et al., 2012). Such analysis could reveal whether the *Z. tritici* septins and cytoskeletal organelles regulate the polymorphic growth and pre-penetration differentiation in *Z. tritici*.

In addition to cell biology, the ability to produce tagged proteins especially transcription factors will enable application of chromatin immunoprecipitation-sequencing (ChIP-seq) to uncover gene regulatory networks in *Z. tritici* (Soyer et al., 2015). Recently in *M. oryzae*, tagged proteins and ChIP-seq enabled identification of more than 140 genes, including several that encode virulence-associated proteins such as chitin synthase and P-type ATPases, which are regulated through calcium mediated signalling by transcription factor CRZ1 (Kim et al., 2010). Similarly, the *Z. tritici* transcription factors cloned by Cairns et al., (2015) could be used to interrogate their downstream targets. Due to their high connectivity (centrality) within the regulatory networks, the transcription factors are emerging as potential drug targets (Grivas et al., 2011). Therefore, transcriptional network analysis in *Z. tritici* is another avenue for identification of novel fungicide targets. Thus the expression vectors reported in the current study (Chapter 4)(Sidhu et al., 2015b) provide a new mode of functional genomics though gain of function analysis and cell biology. As a result, these vectors will facilitate investigations into the role of essential genes and the regulatory networks that fine-tune the expression of *Z. tritici* genes at various stages of STB disease.

### 7.4 Emergence of *Z. tritici* as a model fungal pathogen

*Z. tritici* is one of a few genetically tractable and well studied species out of several economic important plant pathogens in the family *Mycosphaerellaceae* and the order *Dothideales* (Ponomarenko et al., 2011,
Goodwin et al., 2004, Kema et al., 2008). In addition, the life cycle of *Z. tritici* encompasses several interesting biological features such as the exclusive stomatal penetration, temporal host-pathogen interactions, which underpin extended asymptomatic phase of infection, host specificity, polymorphic growth, genome plasticity and rapid effector evolution (Kema et al., 2008, Choi and Goodwin, 2011, Kellner et al., 2014, Mirzadi Gohari et al., 2015, Poppe et al., 2015). These features and increasing availability of genomic resources have meant that *Z. tritici* is increasingly described as a model organism for *Mycosphaerella* pathogens and an emerging model system for studying fungal biology and host-pathogen interactions (Perez-Nadales et al., 2014).

Until recently, the lack of resources for functional genomic analyses withheld exploitation of *Z. tritici* as a model plant pathogen. This bottleneck has been largely addressed by the molecular tools developed in the current study and additional tools previously introduced by Talbot, 2015. In addition to genome sequence of *Z. tritici*, whole genome sequence of its sister species *Z. pseudotritici*, which exclusively infects grasses *Elymus repens* and *Dactylis glomerata*, is also available to the *Mycosphaerella* pathogen research community. Consequently, *Z. tritici* could be exploited as a model for comparative genomics to study genome evolution, speciation and host specialization. The efficient sexual and asexual reproduction of *Z. tritici* has made it an ideal model for studying population and transmission genetics (Stukenbrock et al., 2011). In parallel, the new cell biological capabilities (Steinberg, 2015) and polymorphic growth imply that *Z. tritici* could be valuable organism to study biological basis of fungal development and morphological differentiation. Developmental polymorphism and cell biological tools also make *Z. tritici* an excellent model system to interrogate spatial and temporal protein
organisation and folding as the same proteins could be studied in various
growth morphologies.

The exclusive stomatal penetration strategy makes *Z. tritici* an attractive
organism for studying evolution of chemotropic and thigmotropic sensing
alongside the evolution of direct and indirect host invasion strategies among
plant pathogens. Furthermore, the extended latent phase involves an array of
cross communications (Rudd et al., 2015), extracellular growth and a plethora
of SSPs (do Amaral et al., 2012) are multiple factors of the ploy used by *Z. tritici*
to complete its life cycle in the face of host immunity. This interesting infection
strategy makes the Wheat - *Z. tritici* pathosystem as an excellent model for
studying molecular cross-talks that lead to successful biotrophic, necrotrophic
and hemibiotrophic interactions. Although role of the SPPs, especially the
rapidly evolving positively selected proteins, is largely unknown (Stukenbrock,
2013, Poppe et al., 2015, Mirzadi Gohari et al., 2015), however, the fine-tuned
infection-stage specific expression suggests that these may potentially
modulate the host defences (Rudd et al., 2015). Therefore, *Z. tritici* is an
excellent model for investigating the role of genomic plasticity and selection
pressures imposed by modern agriculture on effector evolution and effector
mediated modulation of host defences. The functional redundancy could be
overcome through inactivation or overexpression of multiple SSP coding genes
using the molecular tools reported in the current study. Subsequently, the *Z.
tritici* mutants can be phenotypically screened by using the *in planta* infection
model (Fones et al., 2015) and digital quantification technique (Stewart and
McDonald, 2014), Overall, the molecular tools reported in the current study will
complement the existing genomic resources and will serve to unleash the full
potential of *Z. tritici* as a model fungal plant pathogen.
7.5 Implications of dispensability of ICL1 in *Z. tritici* on the biology of *Mycosphaerella* pathogens

The glyoxylate and the TCA cycles enable pathogens to utilise the energy reserves stored in the form of trehalose, glycogen, lipids and carbohydrates in order to overcome carbon deficiency stress during pre-penetration stages of infection (Dunn et al., 2009). Consistent with fungal pathogen *S. nodorum* (Solomon et al., 2004), the requirement of the glyoxylate cycle and particularly MLS enzyme in *Z. tritici* (Chapter 6) has verified that indeed the metabolism of stored lipids is likely to provide energy for fungal growth during early stages of STB infection (Rudd et al., 2015). In contrast to foliar pathogens *L. maculans* and *M. oryzae* (Wang et al., 2003, Idnurm and Howlett, 2002), dispensability of the second glyoxylate cycle enzyme ICL in *Z. tritici* indicates towards independent evolution of metabolic pathways to complement the variation in their life cycles and infection strategies. Such lifestyles specific evolutionary variations are evident from the fact that lipids and triacylglycerols are dominant energy reservoir in *Z. tritici* and *S. nodorum* (Keon et al., 2007, Rudd et al., 2015, Solomon et al., 2004), while *M. oryzae* relies predominantly on glycogen for energy (Thines et al., 2000). Lipids and triacylglycerols store six times more energy as compared to glycogen, hence lipids are selected through evolution as dominant energy reservoirs (Berg et al., 2002). *M. oryzae* rapidly gains access to the nutrients within the host-cytosol thus it appears that glycogen can suffice energy requirement for short-term survival from penetration to host cell death. On the other hand, *Z. tritici* has to survive the lengthy asymptomatic phase of infection and as a result has evolved to store and utilise lipids as more concentrated form of energy reserve. Therefore, fungal life cycle related variations such as direct penetration versus
stomatal invasion and extracellular colonisation are likely to underpin variation in types of energy reserves. Consequently, evolutionary variation in the assimilation or utilisation pathways is likely to be the cause of dispensability and requirement of glyoxylate cycle enzymes in *Z. tritici*.

Similar to *Z. tritici*, the exclusively extracellular fungal growth and lengthy asymptomatic phases of infection are a characteristic features of other *Mycosphaerella* pathogens such as *M. fijiensis*, *M. pinodes*, *M. musicola*, *M. brassicicola* and *M. nubilosa*. Therefore, the link between lipid utilisation, the glyoxylate cycle and fungal virulence may also be conserved in these *Mycosphaerella* species. In *M. fijiensis*, various proteins involved in metabolism and biosynthesis of trehalose lipids were up regulated at pre-penetration and sporulation stages of infection, respectively (Portal et al., 2011). Trehalose biosynthesis and metabolism is crucial for virulence of various fungal pathogens (Divon and Fluhr, 2007) including *S. nodorum* (Lowe et al., 2009). Furthermore, similar to model *Mycosphaerella* species *Z. tritici* (Rudd et al., 2015)(Chapter 6), lipid metabolism and the glyoxylate cycle are suggested to support the growth of *M. fijiensis* in carbon deficient environments (Churchill, 2011). Therefore, consistent with *S. nodorum* and *Z. tritici*, *M. fijiensis* appears to rely on the glyoxylate cycle for lipid utilisation in order to survive during the extended asymptomatic phase of black sigatoka infection in banana.

Unlike *M. fijiensis*, currently there is a lack of comprehensive genomics, transcriptomics and metabolomics data in case of other economically important *Mycosphaerella* pathogens. As a result, it is not possible to speculate on requirement or dispensability of the glyoxylate cycle for normal virulence of these pathogens. While evolutionary relationship, genome sequences and
similar lifecycles imply that nutrition acquisition mechanisms may be widely conserved among *Mycosphaerella* pathogens, however dispensability of *Z. tritici* ICL1 suggests that genomic presence and expression of a protein coding gene or a pathway does not verify its role in fungal virulence. This is also evident from dispensability of the glyoxylate cycle gene MLS1 (personal communications with Dr. Mick Kershaw and Professor N. J. Talbot) and several other *M. oryzae* genes, which encode proteins involved in various catabolic processes including molybdate, galactose, and glucosamine metabolism (Fernandez et al., 2014). Overall, dispensability of *Z. tritici* ICL1 has reinforced that not all the components or the metabolic pathways encoded by the genome of a pathogen are likely to contribute to fungal virulence (Fernandez et al., 2014). As a result, the nutrient acquisition pathways need to be characterised to uncover the role of glyoxylate cycle and dispensability or requirement of ICL activity for virulence of other *Mycosphaerella* pathogens. In addition to revealing whether the dispensability of the glyoxylate cycle is conserved among other *Mycosphaerella* pathogens, such analyses will assist in understanding the ongoing pathogen evolution and their trophic relationships with hosts.

**7.6 Impact of the glyoxylate cycle requirement on the overall model of stealth pathogenesis by *Z. tritici***

The current study is the first to provide conclusive evidence that *Z. tritici* is likely to rely on stored lipids as a source of energy to fuel infectious fungal growth during early stages of STB infection (Chapter 6). Hence, the functional glyoxylate cycle, especially the MLS enzyme activity, is required for full virulence of *Z. tritici*. The reliance on internal reserves implies that hypothetical hemibiotrophic lifestyle of *Z. tritici* must be to be re-examined to refine the
overall model of the stealth pathogenesis during the asymptomatic phase of *Z. tritici* infection. Based on the findings presented in Chapter 6, a new model has emerged for the early stages of infection by *Z. tritici*. This model fits with the absence of biotrophic feeding (Orton et al., 2011) and the lengthy latent period of necrotrophic life style of *Z. tritici* (Rudd, 2015). The new model of infection dictates that the lipid rich *Z. tritici* spores land on the nutrient poor surfaces of the wheat leaves and initiate germination to invade and colonise the leaf through stomata. To produce energy for germination and hyphal growth, the highly concentrated energy reserves in the form of stored lipids get metabolised through β-oxidation (Rudd et al., 2015) and resulting acetate skeletons get channelled into the glyoxylate cycle and subsequently into gluconeogenesis to produce glucose (Chapter 6).

Once inside the leaf, *Z. tritici* hyphae grow and colonise the apoplastic cavities. The fungal growth remains minimal and is sustained by the energy produced from lipid metabolism and without feeding on the nutrient poor apoplastic fluid (Keon et al., 2007). Similar to infection of tomato by *C. fulvum* (Thomma et al., 2005) various membrane spanning hexose transporters are up regulated at this early stage of asymptomatic phase (Yang et al., 2013). This suggests that *Z. tritici* could be surviving on hexose monomers such as glucose imported from the host. However, in comparison to the wild type strain, the *Z. tritici* glyoxylate mutants showed impaired growth on wheat leaf agar, which was expected to be rich in apoplastic and cytosolic contents (Figure 6.5 and Figure 6.6). This illustrated that contents of the apoplastic fluid alone are insufficient to support normal fungal growth, unless glucose is synthesized through gluconeogenesis and glyoxylate cycle or otherwise exogenously supplied (Chapter 6 Section 6.8). Around 4 DAI, the midday point within the
asymptomatic phase, the glyoxylate cycle genes show a severe decline in expression potentially due to exhaustion of lipid reserves or through carbon catabolic repression. Thus, lipid metabolism and glyoxylate cycle serves to maintain minimal growth of Z. tritici, at least until the middle of the asymptomatic phase.

During the midway point in the asymptomatic phase, elevated expression of PCWDEs suppression suggests that host cell walls are degraded in order to release sugar monomers (Brunner et al., 2013). Subsequently, these sugars are imported into Z. tritici by the trans-membrane transporters (Yang et al., 2013, Kellner et al., 2014, Rudd et al., 2015). This release of host cell wall sugars and cytosolic electrolytes from early stage host cell death alters the nutrient content of the apoplastic fluid (Keon et al., 2007, Shetty et al., 2009). The escalation of host cell death leads on to transition to the symptomatic phase, which corresponds with rapid host cell death and availability of sugars fuels extensive fungal growth (Shetty et al., 2009, Keon et al., 2007).

The most important and unanswered question about the pathobiology of Z. tritici is “Why does Z. tritici need a lengthy asymptomatic phase before switching to the necrotrophic life style? The current research has partially contributed to answering this question by revealing that the glyoxylate cycle is key mechanism of nutrient acquisition from lipid metabolism in Z. tritici. Although stored lipid reserves are concentrated and rich energy source (Berg et al., 2002), however these finite and continuously reducing reserves are relatively poor when compared to sugars and cytosolic contents which get released following the host cell death. Additionally, temporal and metabolic restrictions limit the speed and efficiency of energy release from these stored
reserves (Berg et al., 2002). Therefore, limited or slow supply of lipid-derived energy could be one cause of slow invasive hyphal growth. Moreover, early detection of Z. tritici and activation of the host defences (Shetty et al., 2009, Orton et al., 2011) imply that fungus has to grow while evading and subverting host defences (Marshall et al., 2011, Lee et al., 2014). Thus fungus growth may further slowed down by the barrage of host defences. Therefore, Z. tritici appears to balance resource allocation to maintain minimal growth alongside systematic erosion of the host defences through waves of secreted effectors (Mirzadi Gohari et al., 2015, Rudd et al., 2015). Subsequently a tipping point is reached at which Z. tritici recognises that it has achieved sufficient suppression of host defences (O'Driscoll et al., 2014, Rudd et al., 2015), spatial host colonisation (Shetty et al., 2007, Shetty et al., 2003, Kema et al., 1996) and release of host nutrients including sugars and cytosolic contents (Shetty et al., 2009, Keon et al., 2007, Rudd et al., 2015). At this stage, Z. tritici acknowledges that host defences do not present a threat to completion of fungal lifecycle and consequently this fungus switches to the necrotrophic growth phase. Overall, it appears that length of asymptomatic phase is determined by limited availability of fungal nutrition, and dynamic exchanges involving attack and defence between wheat and Z. tritici.

7.7 Summary

In summary, the current study has reported the development and utilisation of invaluable molecular tools to reveal novel biological information about the trophic nature and stealth pathogenesis of Z. tritici. In future, these molecular tools will open new opportunities to interrogate the biology of Wheat-Z. tritici interactions. By facilitating the large-scale functional genomic screens,
these tools will enable Z. tritici research community to answer long-standing questions regarding the biology of this pathogen. Alongside, the ability to functionally characterise essential genes would be useful in identification of new drug targets for development novel fungicides to combat Z. tritici. Furthermore, the requirement of the glyoxylate cycle and lipid utilisation for virulence of Z. tritici on wheat has provided the conclusive evidence to suggest that Z. tritici is a necrotrophic pathogen. In addition, the virulence related role of the glyoxylate cycle in Z. tritici implies that the druggable components within the regulatory and metabolic pathways associated with the glyoxylate cycle could be exploited as potential fungicide targets in Z. tritici. Overall, these molecular tools and the biological significance of the glyoxylate cycle in Z. tritici will prove instrumental in future research and development of new and durable STB disease control measures.


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## Appendix 1

### Functionally characterised *Z. tritici* genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>LEUC</em></td>
<td>3-isopropylmalate dehydrogenase</td>
<td>No phenotypic defect reported</td>
<td>(Adachi et al., 2002)</td>
</tr>
<tr>
<td><em>DGD1</em></td>
<td>Dialkylglycine decarboxylase gene</td>
<td>Mutants unable to utilise 2-methylalanine as a nitrogen source</td>
<td>(Adachi et al., 2003)</td>
</tr>
<tr>
<td><em>SDH-IP</em></td>
<td>Succinate dehydrogenase iron sulfur protein</td>
<td>Mutant were resistance to carboxin fungicide</td>
<td>(Skinner et al., 1998)</td>
</tr>
<tr>
<td><em>ATR1</em></td>
<td>ABC transporter</td>
<td>Up regulated expression in presence of fungicides, No phenotypic defect upon inactivation</td>
<td>(Zwiers and De Waard, 2000, Stergiopoulos et al., 2003)</td>
</tr>
<tr>
<td><em>ATR2</em></td>
<td>ABC transporter</td>
<td>Up regulated expression in presence of fungicides, No phenotypic defect upon inactivation</td>
<td>(Stergiopoulos et al., 2003)</td>
</tr>
<tr>
<td><em>ATR3</em></td>
<td>ABC transporter</td>
<td>No phenotypic defect upon inactivation</td>
<td>(Stergiopoulos et al., 2003)</td>
</tr>
<tr>
<td><em>ATR4</em></td>
<td>ABC transporter</td>
<td>Delayed virulence upon inactivation</td>
<td>(Stergiopoulos et al., 2003)</td>
</tr>
<tr>
<td><em>ATR5</em></td>
<td>ABC transporter</td>
<td>No phenotypic defect upon inactivation</td>
<td>(Stergiopoulos et al., 2003)</td>
</tr>
<tr>
<td><em>HOG1</em></td>
<td>MAPK</td>
<td>Avirulent on wheat leaves, hypersensitive to osmotic stress and fungicides, defects in hyphal growth and melanisation.</td>
<td>(Mehrabi et al., 2006b)</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Function</td>
<td>Notes</td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
<td>----------</td>
<td>-------</td>
</tr>
<tr>
<td>TPK2</td>
<td>Protein kinase catalytic subunit</td>
<td>Increased melanisation, defective hyphal growth, reduced virulence in planta, affects on asexual reproduction and inability to produce pycnidiospores</td>
<td>(Mehrabi and Kema, 2006)</td>
</tr>
<tr>
<td>BCY1</td>
<td>Protein kinase regulatory subunit</td>
<td>Hypersensitive to osmotic stress, reduced melanisation, reduced virulence in planta, affects on asexual reproduction and inability to produce pycnidiospores</td>
<td>(Mehrabi and Kema, 2006)</td>
</tr>
<tr>
<td>SLT2</td>
<td>MAPK</td>
<td>Defects in polarized hyphal growth, reduced melanisation, hypersensitive to fungicides and fungal cell wall degrading enzymes, reduced virulence in planta, affects on asexual reproduction and inability to produce pycnidiospores.</td>
<td>(Mehrabi et al., 2006a)</td>
</tr>
<tr>
<td>FUS3</td>
<td>MAPK</td>
<td>Defects in polarized hyphal growth, reduced melanisation, Avirulence in planta, affects on asexual reproduction and inability to produce pycnidiospores.</td>
<td>(Cousin et al., 2006)</td>
</tr>
<tr>
<td>ATR7</td>
<td>ABC transporter</td>
<td>Inactivation causes defects in iron homeostasis</td>
<td>(Zwiers et al., 2007)</td>
</tr>
<tr>
<td>MFS1</td>
<td>MFS family transporter</td>
<td>Inactivation causes Hypersensitivity to strobilurin fungicides and myco-toxin cercosporin</td>
<td>(Roohparvar et al., 2007, Roohparvar et al., 2008)</td>
</tr>
<tr>
<td>STE7</td>
<td>MAPKK</td>
<td>Avirulence in planta, defects in hyphal growth and melanisation upon inactivation</td>
<td>(Kramer et al., 2009)</td>
</tr>
<tr>
<td>STE11</td>
<td>MAPKKK</td>
<td>Avirulence in planta, defects in hyphal growth and melanization upon inactivation</td>
<td>(Kramer et al., 2009)</td>
</tr>
<tr>
<td>STE12</td>
<td>Transcription factor</td>
<td>Inactivation leads to defects in hyphal growth and severely reduced virulence in planta.</td>
<td>(Kramer et al., 2009)</td>
</tr>
<tr>
<td>STE20</td>
<td>MAPKKKK</td>
<td>Defects in hyphal growth.</td>
<td>(Kramer et al., 2009)</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Phenotypic Effect</td>
<td>Reference</td>
</tr>
<tr>
<td>-------</td>
<td>--------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>STE50</td>
<td>MAPK adapter protein</td>
<td>Defects in hyphal growth and melanization, reduced virulence in planta</td>
<td>(Kramer et al., 2009)</td>
</tr>
<tr>
<td>GPA1</td>
<td>G-protein α subunit 1</td>
<td>Elevated hyphal growth, reduced virulence in planta</td>
<td>(Mehrabi et al., 2009)</td>
</tr>
<tr>
<td>GPA2</td>
<td>G-protein α subunit 2</td>
<td>No phenotypic defect observed</td>
<td>(Mehrabi et al., 2009)</td>
</tr>
<tr>
<td>GPA3</td>
<td>G-protein α subunit 3</td>
<td>Elevated in vitro spore growth, reduced cellular cyclic adenosine monophosphate (c-AMP) content, reduced virulence in planta</td>
<td>(Mehrabi et al., 2009)</td>
</tr>
<tr>
<td>GPB1</td>
<td>G-protein β subunit 1</td>
<td>Elevated anastomosis, reduced intracellular c-AMP content, reduced virulence in planta</td>
<td>(Mehrabi et al., 2009)</td>
</tr>
<tr>
<td>MgNLP</td>
<td>SSP, Necrosis and ethylene inducing peptide like protein (NLP) family</td>
<td>No phenotypic defect observed</td>
<td>(Motteram et al., 2009)</td>
</tr>
<tr>
<td>ALG2</td>
<td>α-1,2- mannosyltransferase</td>
<td>Defects in hyphal growth, hypersensitivity to cell wall stress agent Calcofluor white, production of hypo glycosylated and mis-folded proteins.</td>
<td>(Motteram et al., 2011)</td>
</tr>
<tr>
<td>SDHB</td>
<td>Succinate dehydrogenase subunit B</td>
<td>Resistance against fungicide carboxin, increased virulence in planta</td>
<td>(Bowler et al., 2010, Scalliet et al., 2012)</td>
</tr>
<tr>
<td>TUB1</td>
<td>β-tubulin</td>
<td>Resistance against fungicide benomyl</td>
<td>(Bowler et al., 2010)</td>
</tr>
<tr>
<td>KU70</td>
<td>Non homologous end joining DNA break repair</td>
<td>Deletion increases the frequency of homologous recombination, Mild increase in mutagen sensitivity</td>
<td>(Bowler et al., 2010, Sidhu et al., 2015a)</td>
</tr>
<tr>
<td>1LysM</td>
<td>SSP, Lysin (LysM) domain containing effector protein</td>
<td>No phenotypic defect observed</td>
<td>(Marshall et al., 2011)</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Function</td>
<td>Reference</td>
</tr>
<tr>
<td>------------</td>
<td>--------------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>3LysM</td>
<td>SSP, Lysin (LysM) domain containing effector protein</td>
<td>Avirulence <em>in planta</em>, inability to penetrate host, inability to produce asexual pycnidiospores</td>
<td>(Marshall et al., 2011)</td>
</tr>
<tr>
<td>CC1</td>
<td>C-type cyclin</td>
<td>Defects in hyphal growth, increased melanisation, <em>in vitro</em> stress resistance, reduced virulence <em>in planta</em></td>
<td>(Choi and Goodwin, 2011a)</td>
</tr>
<tr>
<td>VE1</td>
<td>Velvet gene family member</td>
<td>Defects in hyphal growth, reduced melanisation, hypersensitive to osmotic stress, reduced hydrophobicity, altered photo-perception</td>
<td>(Choi and Goodwin, 2011b)</td>
</tr>
<tr>
<td>WOR1</td>
<td>Transcription factor</td>
<td>Defects <em>in vitro</em> spore growth, reduced virulence, transcriptional regulation of secretome</td>
<td>(Gohari et al., 2014)</td>
</tr>
<tr>
<td>KU80</td>
<td>Non homologous end joining DNA break repair</td>
<td>Deletion increases the frequency of homologous recombination, Mild increase in mutagen sensitivity</td>
<td>(Sidhu et al., 2015a)</td>
</tr>
<tr>
<td>BGS1</td>
<td>Cell wall β-(1,3)-glucan synthase</td>
<td>Essential for growth and cell wall stress resistance</td>
<td>(Marchegiani et al., 2015)</td>
</tr>
<tr>
<td>Mycgr3G91471</td>
<td>SSP, Unknown function</td>
<td>No virulence defect</td>
<td>(Rudd et al., 2015)</td>
</tr>
<tr>
<td>Mycgr3G103572</td>
<td>SSP, Unknown function</td>
<td>No virulence defect</td>
<td>(Rudd et al., 2015)</td>
</tr>
<tr>
<td>Mycgr3G107286</td>
<td>SSP, Unknown function</td>
<td>No virulence defect</td>
<td>(Rudd et al., 2015)</td>
</tr>
<tr>
<td>Mycgr3G102792</td>
<td>SSP, Unknown function</td>
<td>No virulence defect</td>
<td>(Rudd et al., 2015)</td>
</tr>
<tr>
<td>Mycgr3G108482</td>
<td>SSP, Unknown function</td>
<td>No virulence defect</td>
<td>(Rudd et al., 2015)</td>
</tr>
<tr>
<td>CBR1</td>
<td>Sterol biosynthesis, Cytochrome b₅ reductase activity</td>
<td>Reduced virulence, severely reduced asexual reproduction, alteration to fatty acid and lipid biosynthesis.</td>
<td>(Derbyshire et al., 2015)</td>
</tr>
<tr>
<td>Gene</td>
<td>Function</td>
<td>Effect in planta</td>
<td>Reference</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------</td>
<td>-------------------------------------------------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>CBR2</td>
<td>Sterol biosynthesis, Cytochrome b&lt;sub&gt;S&lt;/sub&gt; reductase activity</td>
<td>No virulence defect</td>
<td>(Derbyshire et al., 2015)</td>
</tr>
<tr>
<td>CYP-24</td>
<td>Cytochrome P450</td>
<td>No virulence defect</td>
<td>(Derbyshire et al., 2015)</td>
</tr>
<tr>
<td>YAP1</td>
<td>Transcription factor</td>
<td>Required for in vitro oxidative stress tolerance and dispensable for in planta virulence.</td>
<td>(Yang et al., 2015)</td>
</tr>
<tr>
<td>SSP15</td>
<td>SSP, Unknown function</td>
<td>No defect in in planta virulence</td>
<td>(Mirzadi Gohari et al., 2015)</td>
</tr>
<tr>
<td>SSP18</td>
<td>SSP, Unknown function</td>
<td>Minor delay in emergence of symptoms</td>
<td>(Mirzadi Gohari et al., 2015)</td>
</tr>
<tr>
<td>Zt80707</td>
<td>SSP, Unknown function</td>
<td>Reduced in planta virulence, limited asexual pycnidiospore production</td>
<td>(Poppe et al., 2015)</td>
</tr>
<tr>
<td>Zt89160</td>
<td>RCC1 domain containing proteins</td>
<td>Increased virulence on wheat leaves</td>
<td>(Poppe et al., 2015)</td>
</tr>
<tr>
<td>Zt103264</td>
<td>Unknown function</td>
<td>Reduced in planta virulence, limited asexual pycnidiospore production</td>
<td>(Poppe et al., 2015)</td>
</tr>
<tr>
<td>Zt110804</td>
<td>Kinase</td>
<td>No defect in in planta virulence</td>
<td>(Poppe et al., 2015)</td>
</tr>
</tbody>
</table>

ABC - ATP binding cassette; MAPK - Mitogen activated protein kinase; MFS - Major facilitator superfamily; MAPKK - Mitogen activated protein kinase kinase; MAPKKK - Mitogen activated protein kinase kinase kinase; MAPKKKK - Mitogen activated protein kinase kinase kinase kinase; G-protein - Guanine nucleotide binding protein; SSP - Small secreted protein.
## Appendix 2

**Primers used in this study**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5’ – 3’</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>2µ-URA3-F</td>
<td>CCGCGGCGCTTTCCACAAACATTGCTCA</td>
<td>To amplify 2 µ origin of replication and <em>URA3</em> selection marker, which were used to construct the vector pC-HYG-YR.</td>
</tr>
<tr>
<td>2µ-URA3-R</td>
<td>CCGCGGTTAGTTTTGCTGGCCGCATCT</td>
<td></td>
</tr>
<tr>
<td>NPTII-F</td>
<td>TGACTTACCTATTCTACCCAAAGCATCCAAATGATTGAACAAGATGGATT</td>
<td>To amplify the <em>nptII</em> resistance gene, which was used to construct the vector pC-G418-YR.</td>
</tr>
<tr>
<td>NPTII-R</td>
<td>CCCCCCCAAGTGTGCCTGGCCTCTACTCATCATCAAATCACCAATGCATGTCGTACGGGGCA</td>
<td></td>
</tr>
<tr>
<td>BAR-F</td>
<td>TCGACTTACCTATTCTACCCAAAGCATCCAAATGAGCCAGAAGACGC</td>
<td>To amplify the <em>bar</em> resistance gene, which was used to construct the vector pC-BAR-YR.</td>
</tr>
<tr>
<td>BAR-R</td>
<td>CCCCCCCAAGTGTGCCTGGCCTCTACTCATCATCAAATCACCAATGCATGTCGTACGGGGCA</td>
<td></td>
</tr>
<tr>
<td>SUR-F</td>
<td>GAGCTCGGTACCAGTTTGTGGGGCGCGCGCGCGGCTCTTGGTGACGGGCA</td>
<td>To amplify the <em>pILV2:ILV2</em> resistance cassette, which was used to construct the vector pC-SUR-YR.</td>
</tr>
<tr>
<td>SUR-R</td>
<td>CCCCCCCAAGTGTGCCTGGCCTCTACTCATCATCAAATCACCAATGCATGTCGTACGGGGCA</td>
<td></td>
</tr>
<tr>
<td>KU70-LF-F</td>
<td>CCTAGGCACCATGTGGGGGGCCCGCGCGCGCGGCTCTGGCCTAGGACGGCA</td>
<td>To amplify left and right flanks of the <em>Z. tritici</em> KU70 gene, which were used to construct the <em>KU70</em> gene deletion vector pC-G418-KU70-KO.</td>
</tr>
<tr>
<td>KU70-LF-R</td>
<td>AACATGTGGAGTGGAGGGTGACCGAGCTCGGGCTGGGAATTGGCCGGCTTGA</td>
<td></td>
</tr>
<tr>
<td>KU70-RF-F</td>
<td>ATTCCAGTGTCTACTGTGCTGGGCTGCAGCTAGGGCTGCGTGGCTCAGGCGGGCA</td>
<td></td>
</tr>
<tr>
<td>KU70-RF-R</td>
<td>AAGGTTGCGATGCGCTAGGCTCACTAGAGGTTGGGTTGGCTGCTGGTGGGTT</td>
<td></td>
</tr>
<tr>
<td>KU80-LF-F</td>
<td>CCTAGGCGACCTGTGGGGGGCCCGCGCGCGCGCGGCTCTGGCCTAGGACGGCA</td>
<td>To amplifier left and right flanks of the <em>KU80</em> gene deletion vectors pC-G418-KU80-KO and pC-SUR-</td>
</tr>
<tr>
<td>KU80-LF-R</td>
<td>AACATGGTGAGTGGAGGGGTACCGAGCTCGTGCTTGA</td>
<td></td>
</tr>
<tr>
<td><strong>Gene</strong></td>
<td><strong>Left Flank</strong></td>
<td><strong>Right Flank</strong></td>
</tr>
<tr>
<td>----------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td><strong>KU80</strong></td>
<td>TGGTATGTCTGGTTT</td>
<td>ATCCCAAGTGCTACTGCACGTCGACCTAGGGGGGATTACAGTTTTTCACGC</td>
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<tr>
<td><strong>SIDC</strong></td>
<td>CTAGGCCACCATGTTGGCCCGGCCTGGCCGATGGAAG</td>
<td>TCAGTCTAGTGCTTCCTCGGCGTCGACCTAGGGGGGATTACAGTTTTTCACGC</td>
</tr>
<tr>
<td><strong>AGS2</strong></td>
<td>GATCCCTCTAGAGTGCAGAATTCGTGCACGACCTAGGGGGGATTACAGTTTTTCACGC</td>
<td>TCAGTCTAGTGCTTCCTCGGCGTCGACCTAGGGGGGATTACAGTTTTTCACGC</td>
</tr>
<tr>
<td><strong>ICL1</strong></td>
<td>CTAGGCCACCATGTTGGCCCGGCCTGGCCGATGGAAG</td>
<td>TCAGTCTAGTGCTTCCTCGGCGTCGACCTAGGGGGGATTACAGTTTTTCACGC</td>
</tr>
<tr>
<td><strong>KU70</strong></td>
<td>GCGGCATGACGAGATGGGGG</td>
<td>AGGATTACAGTTTTTCACGC</td>
</tr>
<tr>
<td><strong>KU80</strong></td>
<td>ACGTGGATGTTGAGTGGCA</td>
<td>AGGATTACAGTTTTTCACGC</td>
</tr>
<tr>
<td>Primer Name</td>
<td>Sequence</td>
<td>Function</td>
</tr>
<tr>
<td>-------------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>KU80-INT-R</td>
<td>TTTGGGAGGGACCTTCTTAA</td>
<td>To confirm replacement of the KU70 and/or KU80 genes by the ptrpC:nptII and/or pILV2:ILV2SUR marker cassettes.</td>
</tr>
<tr>
<td>NPTII-INT-R</td>
<td>AGGTAGCCGGATCAAAGCGTA</td>
<td></td>
</tr>
<tr>
<td>SUR-INT-R</td>
<td>GTCGACGTGAGACATGCAA</td>
<td></td>
</tr>
<tr>
<td>Probe-F</td>
<td>TCGAATCTTCGCGGGTAGAA</td>
<td>To amplify Southern blot probe to confirm targeted deletion of the KU70 and KU80 genes.</td>
</tr>
<tr>
<td>Probe-R</td>
<td>CAGCTATCATTCCATTGATAC</td>
<td></td>
</tr>
<tr>
<td>Probe2-F</td>
<td>CCCATAACACTGCGTTTCA</td>
<td>To amplify Southern blot probe to confirm targeted deletion of the KU70 gene.</td>
</tr>
<tr>
<td>Probe2-R</td>
<td>TCATCGTCCCCGTGAGAGGAG</td>
<td></td>
</tr>
<tr>
<td>S IDC-EXT-F</td>
<td>CTGTAACGGGGCGGATGTAT</td>
<td>To confirm targeted deletion of the SidC gene.</td>
</tr>
<tr>
<td>S IDC-INT-R</td>
<td>CTGCGATGTCCAAAGGTGAGAT</td>
<td></td>
</tr>
<tr>
<td>AGS2-EXT-F</td>
<td>CATCACCAGTGCAAACAGC</td>
<td>To confirm targeted deletion of the AGS2 gene.</td>
</tr>
<tr>
<td>AGS2-INT-R</td>
<td>TCAGCGGCTCATGCATGATG</td>
<td></td>
</tr>
<tr>
<td>ICL1-EXT-F</td>
<td>CAGCGAGTTGTCGGGATG</td>
<td>To confirm targeted deletion of the ICL1 gene.</td>
</tr>
<tr>
<td>ICL1-INT-R</td>
<td>TCCGACGGCTCATGATG</td>
<td></td>
</tr>
<tr>
<td>HPH-INT-R</td>
<td>CGTCAGGACATTTGGGAC</td>
<td>To confirm replacement of either the SidC, AGS2 or ICL1 by the ptrpC:hph marker cassette.</td>
</tr>
<tr>
<td>HPH-INT-R</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Primers used in Chapter 4

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>attB1-EGFP-F</td>
<td>GGGGACAAGTTTGTACAAAAAAGCAGGCTTGGATGGTGAGCAAGGGCGAGGA</td>
<td>To amplify the EGFP ORF for construction of the pENTRY-EGFP entry clone.</td>
</tr>
<tr>
<td>attB1-EGFP-R</td>
<td>GGGGACCACTTTGTACAAGAAAGCTGCTGCTTGGGATGGACAGCTCGTGATGC</td>
<td></td>
</tr>
<tr>
<td>attB1-PRA1-F</td>
<td>GGGGACAAGTTTGTACAAGAAAGCTGCTGCTTGGGATGGACAGCTCGTGATGC</td>
<td>To amplify the PRA1 ORF for construction of the PENTRY-PRA1 entry clone.</td>
</tr>
<tr>
<td>attB1-PRA1-R</td>
<td>GGGGACAAGTTTGTACAAGAAAGCTGCTGCTTGGGATGGACAGCTCGTGATGC</td>
<td></td>
</tr>
<tr>
<td>attB1-SOD1-F</td>
<td>GGGGACAAGTTTGTACAAGAAAGCTGCTGCTTGGGATGGACAGCTCGTGATGC</td>
<td>To amplify the SOD1 ORF for construction of the pENTRY-SOD1 entry clone.</td>
</tr>
<tr>
<td>attB1-SOD1-R</td>
<td>GGGGACAAGTTTGTACAAGAAAGCTGCTGCTTGGGATGGACAGCTCGTGATGC</td>
<td></td>
</tr>
<tr>
<td>LF-KU70-F</td>
<td>TTAATTCCTAGGGCCACCATGTTGGGCGGCGGCCTGC</td>
<td>To amplify the KU70 gene and the left flank for construction of the pYSKH expression vectors.</td>
</tr>
<tr>
<td>LF-KU70-R</td>
<td>GGTGGGTACCGGACTCCGATGCTGAGATCAGCG</td>
<td></td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>KU70-RF-F</td>
<td>AGTTCCATCTTCTGTC</td>
<td>To amplify the right flank of the KU70 gene for construction of the pYSKH expression vectors.</td>
</tr>
<tr>
<td>KU70-RF-R</td>
<td>ACCACCACCACCACCACGTGTGAATTACAGGGTGCGCTGTCCAAGCGGCTG</td>
<td>To amplify the right flank of the KU70 gene for construction of the pYSKH expression vectors.</td>
</tr>
<tr>
<td>MARKER-F</td>
<td>CGCCGGCAATTCGAGCTCGGTACCACCAAA</td>
<td>To amplify either of the ptrpC:bar, ptrpC:nptII, ptrpC:hph or pILV2:ILV2 \textsuperscript{SUR} marker cassettes used to construct the pYSKH expression vectors.</td>
</tr>
<tr>
<td>MARKER-R</td>
<td>TCCCCAACTGGTTCCCCGTCGGCATCTACT</td>
<td>To amplify the right flank of the KU70 gene for construction of the pYSKH expression vectors.</td>
</tr>
<tr>
<td>pZtTEF-F</td>
<td>AGTAGATGCGACCAGCGGACACCGTTGGGACAAAGCAGTCATGACATGAAGCGG</td>
<td>To amplify the Z. tritici promoter pZtTEF used to construct the pYSKH expression vectors.</td>
</tr>
<tr>
<td>pZtTEF-R</td>
<td>TTCTCGTTCAGCTTTTTGTCAAAACTTTGTATGGATTCGGCTGTTGTTGTTGGT</td>
<td>To amplify the Z. tritici promoter pZtTEF used to construct the pYSKH expression vectors.</td>
</tr>
<tr>
<td>pZtATUB-F</td>
<td>AGTAGATGCGACCAGCGGACACCGTTGGGACAAAGCAGTCATGACATGAAGCGG</td>
<td>To amplify the Z. tritici promoter pZtATUB used to construct the pYSKH expression vectors.</td>
</tr>
<tr>
<td>pZtATUB-R</td>
<td>TTCTCGTTCAGCTTTTTGTCAAAACTTTGTATGGATTCGGCTGTTGTTGTTGGT</td>
<td>To amplify the Z. tritici promoter pZtATUB used to construct the pYSKH expression vectors.</td>
</tr>
<tr>
<td>pZtGAPDH-F</td>
<td>AGTAGATGCGACCAGCGGACACCGTTGGGACAAAGCAGTCATGACATGAAGCGG</td>
<td>To amplify the Z. tritici promoter pZtGAPDH used to construct the pYSKH expression vectors.</td>
</tr>
<tr>
<td>pZtGAPDH-R</td>
<td>TTCTCGTTCAGCTTTTTGTCAAAACTTTGTATGGATTCGGCTGTTGTTGTTGGT</td>
<td>To amplify the Z. tritici promoter pZtGAPDH used to construct the pYSKH expression vectors.</td>
</tr>
<tr>
<td>pZtNIA1-F</td>
<td>AGTAGATGCGACCAGCGGACACCGTTGGGACAAAGCAGTCATGACATGAAGCGG</td>
<td>To amplify the Z. tritici promoter pZtNIA1 used to construct the pYSKH expression vectors.</td>
</tr>
<tr>
<td>pZtNIA1-R</td>
<td>TTCTCGTTCAGCTTTTTGTCAAAACTTTGTATGGATTCGGCTGTTGTTGTTGGT</td>
<td>To amplify the Z. tritici promoter pZtNIA1 used to construct the pYSKH expression vectors.</td>
</tr>
<tr>
<td>ccdB-F</td>
<td>ACAAGGTGCTACAAAGAAGC</td>
<td>To amplify the Gateway® cassettes used to construct the pYSKH expression vectors.</td>
</tr>
<tr>
<td>ccdB-R</td>
<td>AAGCCTGCCATGGCTCGAGGTGACTCTAGACAAATTAAAGCCCTGAGCAGC</td>
<td>To amplify the Gateway® cassettes used to construct the pYSKH expression vectors.</td>
</tr>
<tr>
<td>MCS-F</td>
<td>TCTAGAAGTCGCAGCTCGGCGG</td>
<td>To amplify the multiple cloning site used to construct the pYSKH expression vectors.</td>
</tr>
<tr>
<td>MCS-R</td>
<td>CTGTAATTCACACGTGGTG</td>
<td>To amplify the multiple cloning site used to construct the pYSKH expression vectors.</td>
</tr>
<tr>
<td>pYSKH-INT-FWD</td>
<td>ATTAGTTATGTCACGCTTAC</td>
<td>To confirm Integration of the gene over expression cassettes into the mutated KU70 locus in the Δku70 strain HLS1000.</td>
</tr>
<tr>
<td>KU70-EXT-REV</td>
<td>AAGCCTGCCAGCTCAGCAATG</td>
<td>To confirm Integration of the gene over expression cassettes into the mutated KU70 locus in the Δku70 strain HLS1000.</td>
</tr>
</tbody>
</table>
### Primers used in Chapter 5

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pZtNIA-FWD</strong></td>
<td>AGTAGATGCCGACCGGAAACCAGTTGGGGAGAATGCTTCATGAAATGGCC</td>
<td>To amplify the <em>Z. tritici</em> promoter pZtNIA1 used to construct the CPR vector pC-HYG-pZtNIA1-YR.</td>
</tr>
<tr>
<td><strong>pZtNIA-REV</strong></td>
<td>GCATGCGCTGAGTGCAGTCTAGAGATCCTCGGAGGAGCATAGTAA</td>
<td></td>
</tr>
<tr>
<td><strong>ZtBGS1-LF-F</strong></td>
<td>CTAGGCCACCATGTGGCAGCAGCGCCCATACCTCGCAGCGGATATTCAAC</td>
<td>To amplify left and right flanks of the <em>Z. tritici</em> pZtBGS1 promoter, which were used to construct the promoter replacement vector pYSKH101.</td>
</tr>
<tr>
<td><strong>ZtBGS1-LF-R</strong></td>
<td>TCTTTCAATATCAGTTGGGTACCGAGCTCACGCCTGCCAATAACCGC</td>
<td></td>
</tr>
<tr>
<td><strong>ZtBGS1-RF-F</strong></td>
<td>TCTGCACCGGTATATGTCCTCTCCCGCAATGCCCGCCGCAATATCAAC</td>
<td></td>
</tr>
<tr>
<td><strong>ZtBGS1-RF-R</strong></td>
<td>AGCTTGCAATGCGACTGCAGTCTAGAGGAGGCCGAGATGCGCTGGAGTAGTG</td>
<td></td>
</tr>
<tr>
<td><strong>BGS1-EXT-FWD</strong></td>
<td>ACGAGGACTGGCGAGCTTAC</td>
<td>To confirm replacement of the pZtBGS1 by pZtNIA1 promoter.</td>
</tr>
<tr>
<td><strong>BGS1-INT-REV</strong></td>
<td>CTTCGTTTCCAGCAAAACGG</td>
<td></td>
</tr>
<tr>
<td><strong>ZtNIA1-F</strong></td>
<td>AAGGGAACCGCATAGGAA</td>
<td>To analyse the <em>Z. tritici NIA1</em> gene expression by q-RT-PCR analysis.</td>
</tr>
<tr>
<td><strong>ZtNIA1-R</strong></td>
<td>CAGCAGTTCCTCAGGCTGTATG</td>
<td></td>
</tr>
<tr>
<td><strong>EGFP-F</strong></td>
<td>GAAGGGGAATCGAGCTG</td>
<td>To analyse the <em>EGFP</em> gene expression by q-RT-PCR analysis.</td>
</tr>
<tr>
<td><strong>EGFP-R</strong></td>
<td>CACCTTGTAGGCGGTCTT</td>
<td></td>
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</tbody>
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### Primers used in Chapter 6

<table>
<thead>
<tr>
<th>Primer</th>
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</thead>
<tbody>
<tr>
<td><strong>ICL1-F</strong></td>
<td>GGGGACAAAGTTTGTACAAAAAAGCAGGCTTTGATGTCCGGTCCGACGCAGA</td>
<td>To amplify the <em>ICL1</em> ORF for construction of the pENTRY-ICL1 entry clone.</td>
</tr>
<tr>
<td><strong>ICL1-R</strong></td>
<td>GGGGACAAAGTTTGTACAAAAAAGCAGGCTTTGATGTCCGGTCCGACGCAGA</td>
<td></td>
</tr>
<tr>
<td><strong>MCL1-F</strong></td>
<td>GGGGACAAAGTTTGTACAAAAAAGCAGGCTTTGATGTCCGGTCCGACGCAGA</td>
<td>To amplify the <em>MCL1</em> ORF for construction of the pENTRY-MCL1 entry clone.</td>
</tr>
<tr>
<td><strong>MCL1-R</strong></td>
<td>GGGGACAAAGTTTGTACAAAAAAGCAGGCTTTGATGTCCGGTCCGACGCAGA</td>
<td></td>
</tr>
<tr>
<td><strong>MLS1-F</strong></td>
<td>GGGGACAAAGTTTGTACAAAAAAGCAGGCTTTGATGTCCGGTCCGACGCAGA</td>
<td>To amplify the <em>MLS1</em> ORF for construction of the pENTRY-MLS1 entry clone.</td>
</tr>
<tr>
<td><strong>MLS1-R</strong></td>
<td>GGGGACAAAGTTTGTACAAAAAAGCAGGCTTTGATGTCCGGTCCGACGCAGA</td>
<td></td>
</tr>
<tr>
<td>Gene Abbreviation</td>
<td>Primer Sequence</td>
<td>Description</td>
</tr>
<tr>
<td>-------------------</td>
<td>-----------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ICL-LF-F</td>
<td>GAATGCCCCGACATG</td>
<td>To amplify left and right flanks of the <em>Z. tritici ICL1</em> gene, which were used to construct the ICL1 gene deletion vector pC-SUR-ICL1-KO.</td>
</tr>
<tr>
<td>ICL-LF-R</td>
<td>CCTAGGCCACCACATGGTTGGGCCCCGCGCGCTCTGCCTCGCTTCAGTCAATGAGATG</td>
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</tr>
<tr>
<td>ICL-RF-F</td>
<td>CTCTCTTAGAGTCGACATCGAGCAGCTCTGCAGCTCTGACAAATTATTGATGGAAGAA</td>
<td></td>
</tr>
<tr>
<td>ICL-RF-R</td>
<td>AGTCAAGATCTACCAGGTGGAGCTCCTTATTAGCTCTCCTCTGCCACCG</td>
<td></td>
</tr>
<tr>
<td>MCL1-LF-F</td>
<td>CTAGGCCACCACATGGTTGGGCCCCGCGCGCTCTGCCTCGCTTCAGTCAATGAGATG</td>
<td>To amplify left and right flanks of the <em>Z. tritici MCL1</em> gene, which were used to construct the ICL1 gene deletion vector pC-BAR-MCL1-KO.</td>
</tr>
<tr>
<td>MCL1-LF-R</td>
<td>TCTCTCTTAGAGTCGACATCGAGCAGCTCTGCAGCTCTGACAAATTATTGATGGAAGAA</td>
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</tr>
<tr>
<td>MCL1-RF-F</td>
<td>GATCCTCTTAGAGTCGACATCGAGCAGCTCTGCAGCTCTGACAAATTATTGATGGAAGAA</td>
<td></td>
</tr>
<tr>
<td>MCL1-RF-R</td>
<td>GTCAAGATCTACCAGGTGGAGCTCCTTATTAGCTCTCCTCTGCCACCG</td>
<td></td>
</tr>
<tr>
<td>MLS1-LF-F</td>
<td>CCTAGGCCACCACATGGTTGGGCCCCGCGCGCTCTGCCTCGCTTCAGTCAATGAGATG</td>
<td>To amplify left and right flanks of the <em>Z. tritici MLS1</em> gene, which were used to construct the MLS1 gene deletion vector pC-HYG-MLS1-KO.</td>
</tr>
<tr>
<td>MLS1-LF-R</td>
<td>TCTCTCTTAGAGTCGACATCGAGCAGCTCTGCAGCTCTGACAAATTATTGATGGAAGAA</td>
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</tr>
<tr>
<td>MLS1-RF-F</td>
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</tr>
<tr>
<td>MLS1-RF-R</td>
<td>GTCAAGATCTACCAGGTGGAGCTCCTTATTAGCTCTCCTCTGCCACCG</td>
<td></td>
</tr>
<tr>
<td>ICL1-EXT-F</td>
<td>CAGCAGGTTTGGGACTGAGTAG</td>
<td>To confirm replacement of the <em>ICL1</em> gene by the <em>pILV2:ILV2SUR</em> marker cassette.</td>
</tr>
<tr>
<td>ICL1-INT-R</td>
<td>TCCGACGCGCTCAGTACATTG</td>
<td></td>
</tr>
<tr>
<td>SUR-INT-R</td>
<td>GTCGAGCAGGACAGCATGCAA</td>
<td></td>
</tr>
<tr>
<td>MCL1-EXT-F</td>
<td>ACCTCCTCGCCAGGAGGAGCC</td>
<td>To confirm replacement of the <em>MCL1</em> gene by the <em>ptrpC:bar</em> marker cassette.</td>
</tr>
<tr>
<td>MCL1-INT-R</td>
<td>TCGAATATCCTTGCTCGGC</td>
<td></td>
</tr>
<tr>
<td>BAR-INT-R</td>
<td>CTAAATCTCCTGGTACGGGCA</td>
<td></td>
</tr>
<tr>
<td>MLS1-EXT-F</td>
<td>GTCCCTGTCGACGATCTCAACAGGGA</td>
<td>To confirm replacement of the <em>MLS1</em> gene by the</td>
</tr>
<tr>
<td>MLS1-INT-R</td>
<td>TGAAGACGTCCAGGATCAACG</td>
<td><em>ptrpC:hph</em> marker cassette.</td>
</tr>
<tr>
<td>-----------------</td>
<td>---------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>HYG-INT-R</td>
<td>TAAGATCGGCCGCAGCGATC</td>
<td></td>
</tr>
</tbody>
</table>
# Appendix 3

## Details of the Gateway® compatible *Z. tritici* expression vectors

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Marker cassette (Selection agent)</th>
<th><em>Z. tritici</em> promoter</th>
<th>Gateway cassette/tag</th>
<th>Components of TDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYSKH1</td>
<td><em>ptrpC:bar</em> (Glufosinate ammonium)</td>
<td><em>pZtTEF</em></td>
<td><em>attR1:ccdB:aatR2</em> No tag</td>
<td>LB-KU70LF-KU70-*ptrpC:bar-*pZtTEF-attR1:ccdB:aatR2-T-KU70RF-RB</td>
</tr>
<tr>
<td>pYSKH2</td>
<td><em>ptrpC:nptII</em> (Geneticin)</td>
<td><em>pZtTEF</em></td>
<td><em>attR1:ccdB:aatR2</em> No tag</td>
<td>LB-KU70LF-KU70-*ptrpC:nptII-*pZtTEF-attR1:ccdB:aatR2-T-KU70RF-RB</td>
</tr>
<tr>
<td>pYSKH3</td>
<td><em>ptrpC:hph</em> (Hygromycin)</td>
<td><em>pZtTEF</em></td>
<td><em>attR1:ccdB:aatR2</em> No tag</td>
<td>LB-KU70LF-KU70-*ptrpC:hph-*pZtTEF-attR1:ccdB:aatR2-T-KU70RF-RB</td>
</tr>
<tr>
<td>pYSKH4</td>
<td><em>pILV2:ILV2&lt;sub&gt;SUR&lt;/sub&gt;</em> (Chlorimuron ethyl)</td>
<td><em>pZtGAPDH</em></td>
<td><em>attR1:ccdB:aatR2</em> No tag</td>
<td>LB-KU70LF-KU70-*pILV2:ILV2&lt;sub&gt;SUR&lt;/sub&gt;*pZtGAPDH-attR1:ccdB:aatR2-T-KU70RF-RB</td>
</tr>
<tr>
<td>pYSKH5</td>
<td><em>ptrpC:bar</em> (Glufosinate ammonium)</td>
<td><em>pZtGAPDH</em></td>
<td><em>attR1:ccdB:aatR2</em> No tag</td>
<td>LB-KU70LF-KU70-*ptrpC:bar-*pZtGAPDH-attR1:ccdB:aatR2-T-KU70RF-RB</td>
</tr>
<tr>
<td>pYSKH6</td>
<td><em>ptrpC:nptII</em> (Geneticin)</td>
<td><em>pZtGAPDH</em></td>
<td><em>attR1:ccdB:aatR2</em> No tag</td>
<td>LB-KU70LF-KU70-*ptrpC:nptII-*pZtGAPDH-attR1:ccdB:aatR2-T-KU70RF-RB</td>
</tr>
<tr>
<td>pYSKH7</td>
<td><em>ptrpC:hph</em> (Hygromycin)</td>
<td><em>pZtGAPDH</em></td>
<td><em>attR1:ccdB:aatR2</em> No tag</td>
<td>LB-KU70LF-KU70-*ptrpC:hph-*pZtGAPDH-attR1:ccdB:aatR2-T-KU70RF-RB</td>
</tr>
<tr>
<td>pYSKH8</td>
<td><em>pILV2:ILV2&lt;sub&gt;SUR&lt;/sub&gt;</em> (Chlorimuron ethyl)</td>
<td><em>pZtGAPDH</em></td>
<td><em>attR1:ccdB:aatR2</em> No tag</td>
<td>LB-KU70LF-KU70-*pILV2:ILV2&lt;sub&gt;SUR&lt;/sub&gt;*pZtGAPDH-attR1:ccdB:aatR2-T-KU70RF-RB</td>
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<tr>
<td>pYSKH9</td>
<td><em>ptrpC:bar</em> (Glufosinate ammonium)</td>
<td><em>pZtATUB</em></td>
<td><em>attR1:ccdB:aatR2</em> No tag</td>
<td>LB-KU70LF-KU70-*ptrpC:bar-*pZtATUB-attR1:ccdB:aatR2-T-KU70RF-RB</td>
</tr>
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<td><em>pZtATUB</em></td>
<td><em>attR1:ccdB:aatR2</em> No tag</td>
<td>LB-KU70LF-KU70-*ptrpC:nptII-*pZtATUB-attR1:ccdB:aatR2-T-KU70RF-RB</td>
</tr>
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<td>pYSKH11</td>
<td><em>ptrpC:hph</em> (Hygromycin)</td>
<td><em>pZtATUB</em></td>
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<td>LB-KU70LF-KU70-*ptrpC:hph-*pZtATUB-attR1:ccdB:aatR2-T-KU70RF-RB</td>
</tr>
<tr>
<td>pYSKH12</td>
<td><em>pILV2:ILV2&lt;sub&gt;SUR&lt;/sub&gt;</em> (Chlorimuron ethyl)</td>
<td><em>pZtATUB</em></td>
<td><em>attR1:ccdB:aatR2</em> No tag</td>
<td>LB-KU70LF-KU70-*pILV2:ILV2&lt;sub&gt;SUR&lt;/sub&gt;*pZtATUB-attR1:ccdB:aatR2-T-KU70RF-RB</td>
</tr>
<tr>
<td>pYSKH13</td>
<td><em>ptrpC:bar</em> (Glufosinate ammonium)</td>
<td><em>pZtTEF</em></td>
<td><em>attR1:ccdB:aatR2</em> EGFP</td>
<td>LB-KU70LF-KU70-*ptrpC:bar-*pZtTEF-attR1:ccdB:aatR2-EGFP-T-KU70RF-RB</td>
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<td>pYSKH14</td>
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<td><em>pZtTEF</em></td>
<td><em>attR1:ccdB:aatR2</em> EGFP</td>
<td>LB-KU70LF-KU70-*ptrpC:nptII-*pZtTEF-attR1:ccdB:aatR2-EGFP-T-KU70RF-RB</td>
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<tr>
<td>pYSKH15</td>
<td><em>ptrpC:hph</em> (Hygromycin)</td>
<td><em>pZtTEF</em></td>
<td><em>attR1:ccdB:aatR2</em> EGFP</td>
<td>LB-KU70LF-KU70-*ptrpC:hph-*pZtTEF-attR1:ccdB:aatR2-EGFP-T-KU70RF-RB</td>
</tr>
</tbody>
</table>
pYSKH16  
**pILV2:ILV2**\textsuperscript{SUR} (Chlorimuron ethyl)  
\textit{pZtTEF}  
\textit{attR1:ccdB:aatR2:EGFP}  
\textit{LB-KU70LF-KU70-pILV2:ILV2**\textsuperscript{SUR}-pZtTEF-attR1:ccdB:aatR2:EGFP-T-KU70RF-RB}

pYSKH17  
\textit{ptrpC:bar} (Glufosinate ammonium)  
\textit{pZtGAPDH}  
\textit{attR1:ccdB:aatR2:EGFP}  
\textit{LB-KU70LF-KU70-ptrpC:bar-pZtGAPDH-attR1:ccdB:aatR2:EGFP-T-KU70RF-RB}

pYSKH18  
\textit{ptrpC:nptII} (Geneticin)  
\textit{pZtGAPDH}  
\textit{attR1:ccdB:aatR2:EGFP}  
\textit{LB-KU70LF-KU70-ptrpC:nptII-pZtGAPDH-attR1:ccdB:aatR2:EGFP-T-KU70RF-RB}

pYSKH19  
\textit{ptrpC:hph} (Hygromycin)  
\textit{pZtGAPDH}  
\textit{attR1:ccdB:aatR2:EGFP}  
\textit{LB-KU70LF-KU70-ptrpC:hph-pZtGAPDH-attR1:ccdB:aatR2:EGFP-T-KU70RF-RB}

pYSKH20  
**pILV2:ILV2**\textsuperscript{SUR} (Chlorimuron ethyl)  
\textit{pZtGAPDH}  
\textit{attR1:ccdB:aatR2:EGFP}  
\textit{LB-KU70LF-KU70-pILV2:ILV2**\textsuperscript{SUR}-pZtGAPDH-attR1:ccdB:aatR2:EGFP-T-KU70RF-RB}

pYSKH21  
\textit{ptrpC:bar} (Glufosinate ammonium)  
\textit{pZtATUB}  
\textit{attR1:ccdB:aatR2:EGFP}  
\textit{LB-KU70LF-KU70-ptrpC:bar-pATUB-attR1:ccdB:aatR2:EGFP-T-KU70RF-RB}

pYSKH22  
\textit{ptrpC:nptII} (Geneticin)  
\textit{pZtATUB}  
\textit{attR1:ccdB:aatR2:EGFP}  
\textit{LB-KU70LF-KU70-ptrpC:nptII-pZtATUB-attR1:ccdB:aatR2:EGFP-T-KU70RF-RB}

pYSKH23  
\textit{ptrpC:hph} (Hygromycin)  
\textit{pZtATUB}  
\textit{attR1:ccdB:aatR2:EGFP}  
\textit{LB-KU70LF-KU70-ptrpC:hph-pZtATUB-attR1:ccdB:aatR2:EGFP-T-KU70RF-RB}

pYSKH24  
**pILV2:ILV2**\textsuperscript{SUR} (Chlorimuron ethyl)  
\textit{pZtATUB}  
\textit{attR1:ccdB:aatR2:EGFP}  
\textit{LB-KU70LF-KU70-pILV2:ILV2**\textsuperscript{SUR}-pZtATUB-attR1:ccdB:aatR2:EGFP-T-KU70RF-RB}

pYSKH25  
\textit{ptrpC:bar} (Glufosinate ammonium)  
\textit{pZtNIA1}  
\textit{attR1:ccdB:aatR2:No tag}  
\textit{LB-KU70LF-KU70-ptrpC:bar-pZtNIA1-attR1:ccdB:aatR2-T-KU70RF-RB}

pYSKH26  
\textit{ptrpC:nptII} (Geneticin)  
\textit{pZtNIA1}  
\textit{attR1:ccdB:aatR2:No tag}  
\textit{LB-KU70LF-KU70-ptrpC:nptII-pZtNIA1-attR1:ccdB:aatR2-T-KU70RF-RB}

pYSKH27  
\textit{ptrpC:hph} (Hygromycin)  
\textit{pZtNIA1}  
\textit{attR1:ccdB:aatR2:No tag}  
\textit{LB-KU70LF-KU70-ptrpC:hph-pZtNIA1-attR1:ccdB:aatR2-T-KU70RF-RB}

pYSKH28  
**pILV2:ILV2**\textsuperscript{SUR} (Chlorimuron ethyl)  
\textit{pZtNIA1}  
\textit{attR1:ccdB:aatR2:No tag}  
\textit{LB-KU70LF-KU70-pILV2:ILV2**\textsuperscript{SUR}-pZtNIA1-attR1:ccdB:aatR2-T-KU70RF-RB}

pYSKH29  
\textit{ptrpC:bar} (Glufosinate ammonium)  
\textit{pZtNIA1}  
\textit{attR1:ccdB:aatR2:EGFP}  
\textit{LB-KU70LF-KU70-ptrpC:bar-pZtNIA1-attR1:ccdB:aatR2:EGFP-T-KU70RF-RB}

pYSKH30  
\textit{ptrpC:nptII} (Geneticin)  
\textit{pZtNIA1}  
\textit{attR1:ccdB:aatR2:EGFP}  
\textit{LB-KU70LF-KU70-ptrpC:nptII-pZtNIA1-attR1:ccdB:aatR2:EGFP-T-KU70RF-RB}

pYSKH31  
\textit{ptrpC:hph} (Hygromycin)  
\textit{pZtNIA1}  
\textit{attR1:ccdB:aatR2:EGFP}  
\textit{LB-KU70LF-KU70-ptrpC:hph-pZtNIA1-attR1:ccdB:aatR2:EGFP-T-KU70RF-RB}

pYSKH32  
**pILV2:ILV2**\textsuperscript{SUR} (Chlorimuron ethyl)  
\textit{pZtNIA1}  
\textit{attR1:ccdB:aatR2:EGFP}  
\textit{LB-KU70LF-KU70-pILV2:ILV2**\textsuperscript{SUR}-pZtNIA1-attR1:ccdB:aatR2:EGFP-T-KU70RF-RB}

The complete nucleotide sequences and genetic maps of these vectors are publically available at the link below  
https://www.dropbox.com/sh/k6df896pfb660nk/AAB6XpWtiX5KoZaNP0m0rHba?dl=0
Appendix 4

Exploitation of sulfonylurea resistance marker and non-homologous end joining mutants for functional analysis in *Zymoseptoria tritici*


Abstract

The lack of techniques for rapid assembly of gene deletion vectors, paucity of selectable marker genes available for genetic manipulation and low frequency of homologous recombination are major constraints in construction of gene deletion mutants in *Zymoseptoria tritici*. To address these issues, we have constructed ternary vectors for *Agrobacterium tumefaciens* mediated transformation of *Z. tritici*, which enable the single step assembly of multiple fragments via yeast recombinational cloning. The sulfonylurea resistance gene, which is a mutated allele of the *Magnaporthe oryzae* ILV2 gene, was established as a new dominant selectable marker for *Z. tritici*. To increase the frequency of homologous recombination, we have constructed *Z. tritici* strains deficient in the non-homologous end joining pathway of DNA double stranded break repair. This was achieved by inactivating the *KU70* and *KU80* genes. Targeted gene deletion frequency increased to more than 85% in both *Z. tritici* ku70 and ku80 null strains, compared to ≤10% seen in wildtype parental strain IPO323. The in vitro growth and *in planta* pathogenicity of the *Z. tritici* ku70 and ku80 null strains were comparable to strain IPO323. Together these molecular tools add significantly to the platform available for genomic analysis through targeted gene deletion or promoter replacements and will facilitate large-scale functional characterization projects in *Z. tritici*.

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1. Introduction

The ascomycete fungal pathogen *Zymoseptoria tritici* causes *Septoria tritici* leaf blotch (STB) in cultivated wheat (*Triticum* species). It is one of the most economically important pathogens of wheat in the Northern Hemisphere and can reduce annual wheat yields by up to 50% if the disease is not controlled (Poley and Thomas, 1991). The pathogen overwinters as ascospores, which are dispersed by wind to initiate polycyclic STB infection in susceptible hosts (Orton et al., 2011). *Z. tritici* ascospores germinate on leaf surfaces and invade the host through stomata and continue to grow within the host tissue for 8–12 days without visible symptoms on the leaf surface (Goodwin et al., 2011). This biotrophic growth is followed by a switch to the necrotrophic growth phase which is characterized by a rapid increase in fungal biomass and emergence of STB symptoms including necrosis, chlorosis and typical brown black pycnidal lesions. To develop new disease control measures, it is crucial to understand the molecular mechanisms that underpin this developmental programme (Orton et al., 2011; Goodwin et al., 2011).

In *Z. tritici*, targeted gene deletion is the main strategy for functional genetic analysis and has been used to characterize various gene families, including members of those encoding secreted effectors (Motteram et al., 2009), mitogen activated protein kinases (Mehrabi et al., 2006a,b) and ATP-binding cassette transporters (Zwiers et al., 2003). *Agrobacterium tumefaciens* mediated transformation (ATMT) is the most widely used method of introducing foreign DNA into the *Z. tritici* genome (Zwiers and De Waard, 2001; Bowler et al., 2010). As is the case for many ascomycete fungi such as *Neurospora crassa* (Ninomiya et al., 2004), *Stagonospora nodorum* (Feng et al., 2012) and *Magnaporthe oryzae* (Kershaw and Talbot, 2009) the gene targeting efficiency in *Z. tritici* is often low and locus dependent (Bowler et al., 2010) and is thus not amenable to a medium or high-throughput functional genomics analysis. Several physical factors such as chromosomal position, chromatin structure and transcriptional state of the target region affects the homologous recombination (HR) mediated integration of transforming DNA (Ninomiya et al., 2004; Feng et al., 2012). The non-homologous end joining (NHEJ) pathway of double strand DNA break repair also has a major impact on the frequency of HR (Walker et al., 2001). In most ascomycete fungi, disruption of
the NHEJ pathway of DNA repair leads to elevated gene targeting efficiency because the HR pathway remains the only functional DNA break repair mechanism resulting in increased frequency of HR between the genome and foreign DNA (Ninomiya et al., 2004; Kershaw and Talbot, 2009). The eukaryotic NHEJ pathway is regulated by a protein complex comprising of the Ku70–Ku80 protein heterodimer and a DNA-dependent protein kinase catalytic subunit (Walker et al., 2001). Thus inactivation of either or both the Ku protein encoding genes leads to the disruption of the NHEJ pathway (Ninomiya et al., 2004).

In addition to low HR, the paucity of available positive selection markers is a significant constraint on large scale reverse genetics in Z. tritici. Three selectable marker genes, namely the phosphinothricin acetyltransferase encoding bar gene isolated from Streptomyces hygroscopicus (Thompson et al., 1987), the neomycin phosphotransferase II encoding nptII gene and the hygromycin phosphotransferase encoding hph gene (Gritz and Davies, 1983), both isolated from Escherichia coli, which confer resistance against glufosinate ammonium, geneticin and hygromycin B respectively, are routinely used in genetic manipulation of Z. tritici (Payne et al., 1998; Bowler et al., 2010; Zwiers and De Waard, 2001; Kramer et al., 2009). In addition, the carbendazim and carboxin resistant alleles of the β-tubulin encoding TUB1 and succinate dehydrogenase subunit B encoding MgsSDBH gene from Z. tritici, respectively were also established as selectable markers (Payne et al., 1998; Bowler et al., 2010). However these markers are rarely used for genetic manipulations of Z. tritici. The mutation leading to carboxin resistance in Z. tritici (Scalliet et al., 2012) and carbendazim resistance in Fusarium graminearum (Zhang et al., 2009) are also known to affect in planta virulence in these organisms. This implies that the host derived marker genes are unsuitable for use in genetic manipulation to investigate traits associated with fungal pathogenicity and virulence. A different resistance marker was used in a previous study to inactivate the Z. tritici ku700 gene (Bowler et al., 2010); therefore, only two markers conferring resistance to hygromycin and glufosinate ammonium remain for use in genetic manipulations of this Z. tritici ku70 null strain. Thus it is difficult to construct triple gene deletion mutants in this background. Such multiple gene deletion strains would be useful for characterizing complex traits associated with fungal infection, as, for example, in the analysis of secreted proteinases in Candida albicans and zinc transporters in Aspergillus fumigatus (Amich et al., 2010; Watts et al., 1998). Auxotrophic markers are also used in genetic manipulation of fungi. However, disruption of amino acid synthesis can affect fungal pathogenesis and therefore the use of auxotrophic markers should be avoided (de Gontijo et al., 2014; Swegard et al., 1998). By increasing the number of selectable markers available for Z. tritici, it should be possible to facilitate the analysis of complex virulence phenotypes.

This report describes the construction and utility of Z. tritici ku70 and ku80 null strains in improving gene-targeting efficiency. The establishment of a sulfonylurea resistance cassette (ILV2ΔOR [GenBank accession AF013601], based on an allele of the Mgnaporter oryzae ILV2 gene, which encodes a variant of the acetalocatate synthase enzyme that confers resistance to chlorimuron ethyl (a sulfonylurea herbicide), as a new dominant selectable marker system for Z. tritici (Valent and Chumley, 1991). In addition we have adapted the A. tumefaciens and E. coli binary vector backbone pCAMBIA-0380 to facilitate yeast recombinational cloning in Saccharomyces cerevisiae (Collopy et al., 2010; Gibson et al., 2008). Taken together these tools add significantly to our ability to genetically manipulate Z. tritici.

2. Materials and methods

2.1. Strains and growth conditions

The Z. tritici wild-type strain IPO323 was purchased from the Fungal Biodiversity Centre, Netherlands (http://www.cls.knaw.nl). The IPO323 genome sequence was accessed at http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html (Goodwin et al., 2011). All reagents and chemicals were purchased from Sigma–Aldrich (Dorset, UK), unless otherwise stated.

All Z. tritici strains used in this study were cultured on Yeast Peptone Dextrose agar (YPD agar, per litre: 10 g yeast extract, 20 g peptone, 20 g dextrose and 20 g agar) or Basal medium (BM per litre: 1.7 g yeast nitrogen base without amino acids or ammonia sulphate (Formedium™, Hunstanton, UK), 2 g asparagine, 1 g NH4NO3, 10 g glucose and 20 g agar, pH to 6.0 with 1 M Na2HPO4) (Yang and Naqui, 2014). The selection agent chlorimuron ethyl referred to as sulfonylurea hereafter (a gift from DuPont, Wilmington, USA) was dissolved in dimethylformamide to make a 2 mg/mL stock solution. Induction Medium agar (IM agar; 10 mM K2HPO4, 10 mM KH2PO4, 2.5 mM NaCl, 2 mM MgSO4, 7H2O, 0.7 mM CaCl2, 10 μM FeSO4, 4 mM (NH4)2SO4, 10 mM Glucose, 40 mM 2-N-morpholino ethosulfonic acid, 0.5% Glycerol (w/v), 15 g agar/litre (w/v); pH to 5.6 with 1 M NaOH) supplemented with 200 μM acetosyringone was used for ATMT. For the mutagen sensitivity screen, Z. tritici strains were inoculated in synthetic complete (SC) broth containing 2.5 μM chloroacetamide (CAM) per litre. After 48–72 h incubation at 30 °C the agar surface. Harvested cell suspensions were enumerated using a haemocytometer and suspensions were adjusted to required concentrations. S. cerevisiae strain BY4741 (MATa, his3, leu2, met15, ura3) was used for plasmid construction via recombinational cloning. A 50 ml YPD culture was inoculated with a single colony and grown at 30 °C and 180 RPM for 12 h to early stationary phase. Cells were harvested and used for standard transformations protocols (Gietz and Woods, 2002). The transformed cells were spread on SC dropout agar lacking uracil (SC-URA agar per litre: 6.9 g yeast nitrogen base with ammonium sulphate without amino acids (Formedium™), 790 mg complete amino acid supplement mixture (Formedium™) and 20 g glucose) and cultures were grown at 18 °C and 200 RPM for 6 days. For microscopy, cultures were grown in SC, Czapek Dox broth (CDB) (Sigma–Aldrich, UK) or Aspergillus minimal media (AMM) broth (Bowler et al., 2010) for 72 h at 18 °C and 120 RPM. For ATMT and the sulfonylurea sensitivity screen, Z. tritici yeast like budding cells were grown on YPD agar plates incubated at 18 °C for 6 days, then harvested by adding sterile water and gently scraping the agar surface. Harvested cell suspensions were enumerated using a haemocytometer and suspensions were adjusted to required concentrations. S. cerevisiae strain BY4741 (MATa, his3, leu2, met15, ura3) was used for plasmid construction via recombinational cloning. A 50 ml YPD culture was inoculated with a single colony and grown at 30 °C and 180 RPM for 12 h to early stationary phase. Cells were harvested and used for standard transformations protocols (Gietz and Woods, 2002). The transformed cells were spread on SC dropout agar lacking uracil (SC-URA agar per litre: 6.9 g yeast nitrogen base with ammonium sulphate without amino acids (Formedium™), 790 mg complete amino acid supplement without uracil (Formedium™), 20 g glucose and 20 g agar) to select uracil prototrophs. Individual prototrophic colonies were identified after 48–72 h incubation at 30 °C. E. coli strain DH5α was used to propagate all plasmids. E. coli cultures were grown at 37 °C in Luria–Bertani (LB) media supplemented with 100 μg/mL kanamycin. A. tumefaciens strain EHA105 was used for the ATMT of Z. tritici. A. tumefaciens cultures were grown at 30 °C in LB or IM broth containing 100 μg/mL kanamycin and 100 μg/mL rifampicin.

2.2. Nucleic acid manipulations

Molecular cloning and DNA manipulations were carried out following standard protocols (Sambrook and Russell, 2001), unless otherwise stated. All PCR primers are shown in Supplementary Table S1. Phusion™ high fidelity polymerase (New England Biolabs (NEB), Herts, UK) was used to PCR amplify fragments for cloning while GoTaq® flexi DNA polymerase (Promega,
Southampton, UK) was used for diagnostic colony PCR. All restriction endonucleases and T4-DNA ligase were purchased from NEB. Recombinant vectors were sequenced using the Sanger sequencing service at Eurofins Genomics (Ebersberg, Germany). Z. tritici genomic DNA was extracted in a Fastprep® cell suspension (Fisher Scientific, Loughborough, UK) using a phenol chloroform extraction protocol (Motteram et al., 2009). Plasmids were extracted from E. coli using the QIAprep spin mini-prep kit (Qiagen, Manchester, UK). Recombinant plasmids were isolated from S. cerevisiae as described by Singh and Weil (2002).

2.3. Vector construction

The A. tumefaciens binary vector pHYG (Motteram et al., 2009) was modified by addition of the S. cerevisiae 2μ origin of replication and the URA3 selection marker to construct ternary vector pHYG-YR (Addgene ID – 61765) (see supplementary materials and methods). The ternary vectors pHYG-SUR-YR (Addgene ID – 61768), pC-HYG-KU70-KO, pC-HYG-KU80-KO and pHYG-KU80-KO (Addgene ID – 61767) in which the hygromycin resistance marker of pHYG-YR was replaced with the markers which confer resistance against sulfonylurea, glufosinate ammonium and genetin, respectively were constructed by yeast recombinational cloning (see supplementary materials and methods). To generate cassettes for inactivation of the KU70 and KU80 genes, left flanks (LF) and right flanks (RF) of each gene were cloned by yeast recombinational cloning on either side of the resistance marker in the vectors pC-G418-YR and pC-SUR-YR to construct pC-G418-KU70-KO, pC-G418-KU80-KO and pC-SUR-KU80-KO (see supplementary materials and methods). Similarly, vectors pHYG-36951-KO, pHYG-72646-KO and pHYG-102083-KO used to inactivate the Z. tritici genes Mycgr3G36951 (encoding a putative non-ribosomal peptide synthetase), Mycgr3G72646 (encoding a putative α-(1.3)-glucan synthase) and Mycgr3G102083 (encoding putative isocitrate lyase) respectively were constructed using vector pHYG-YR and yeast recombinational cloning (see supplementary materials and methods).

2.4. A. tumefaciens mediated transformation of Z. tritici

The vectors pHYG-KU70-KO, pHYG-KU80-KO, pHYG-SUR-YR-KO, pC-HYG-36951-KO, pC-HYG-72646-KO, pC-HYG-102083-KO and pHYG-SUR-YR were introduced into chemically competent cells of A. tumefaciens strain EHA105 (Flowers and Vaillancourt, 2005). ATMT of Z. tritici was carried out with minor modifications to standard protocols (Zwiets and De Waard, 2001; Motteram et al., 2009). Briefly, A. tumefaciens cultures (OD595 = 0.25–0.30) carrying the appropriate vector and a Z. tritici cell suspension (1×10^7 cells/ml in sterile water) were mixed in a ratio of 3:1 and 400 μl of this mixture was spread on nitrocellulose membranes (A.A. Packaging Limited, Preston, UK) placed on IM agar. After 48 h incubation at 25°C, the nitrocellulose discs were transferred, as appropriate for each vector, onto selection plates containing BM agar amended with 10 μg/ml sulfonylurea or YPD agar containing 200 μg/ml geneticin (G418 sodium salt) or hygromycin B and antibiotics (100 μg/ml ampicillin, 100 μg/ml cefotaxime, 100 μg/ml streptomycin and 100 μg/ml timentin). After 14 days putative drug resistant Z. tritici transformants were sub cultured for two rounds of selection on BM agar containing 10 μg/ml sulfonylurea or YPD agar containing 200 μg/ml geneticin or hygromycin and confirmed drug resistant strains were selected for further analysis.

To confirm the functionality of the sulfonylurea resistance cassette, fifteen independent sulfonylurea resistant transformants harbouring the ternary vector pC-SUR-YR were isolated and designated as strains IPO323::SurR^1–15. The genomic integration of the sulfonylurea resistance cassette in strains IPO323::SurR^1–15 was confirmed by diagnostic PCR using primers pSurR^6-FWD and SUR^6-INT-R which amplify a single 558 bp fragment specific to the resistance cassette.

Putative Z. tritici ku70 and ku80 null strains resistant to genetin and harbouring pC-G418-KU70-KO and pC-G418-KU80-KO were isolated. These strains were designated HLS1000 (Aku70:G418^6) and HLS1001 (Aku80:G418^6) respectively. Similarly to demonstrate the application of sulfonylurea resistance cassette as a marker for targeted gene deletion in Z. tritici, sulfonylurea resistant strains transformed with pC-SUR-KU80-KO were isolated and designated HLS1002 (Aku80:SurR^6). To confirm the inactivation of the Z. tritici KU70 and KU80 genes, drug resistant transformants were analysed by diagnostic PCR using primer combinations KU70-EXT-F/KU70-INT-R/G418^6-INT-R, KU80-EXT-F/KU80-INT-R/G418^6-INT-R or KU80-EXT-F/KU80-INT-R/SUR^6-INT-R. To confirm the integration events of deletion vectors into strains HLS1000, HLS1001 and HLS1002, Southern blot analysis was performed as described by Motteram et al. (2009), Sambrook and Russell (2001). Briefly genomic DNA isolated from Z. tritici strains HLS1000, HLS1001 and IPO323 was digested with PvuI and HindII followed by transfer onto nylon membranes. A 152 bp long DIG-DUTP labelled DNA probe was synthesized using PCR primers Probe-F/R which are specific to the genetin resistance cassette (Supplementary Table S1) following the manufacturers’ instruction using PCR DIG probe synthesis kit (Roche Diagnostics Ltd., Burgess Hill, UK). After incubation of the membrane in the probe hybridization solution for 12 h at 65°C, the membrane was washed as described by Motteram et al. (2009) and images were acquired using a GBOX Chemi XX6 imaging system (Syngene, Cambridge, UK). A single hybridization product at 2409 bp and 2887 bp confirmed the deletion of the KU70 and KU80 gene respectively without any events of ectopic integration of the deletion construct. Similarly, another Southern blot analysis was also performed on the genomic DNA isolated from strains HLS1002 and IPO323 which was digested with restriction endonucleases BstEII and SphI. The DIG-DUTP labelled probe specific to the KU80 right flank was synthesized using primers KU80-Probe-F/R (Supplementary Table S1). A single hybridization product at 3792 bp indicated deletion of the KU80 gene in strain HLS1002 while a single hybridization band at 7241 bp indicated the wild type KU80 gene.

To compare gene targeting efficiency in strains HLS1000 and HLS1001 to the wild type strain IPO323, hygromycin resistant strains from each strain background transformed with pC-HYG-36951-KO, pC-HYG-72646-KO and pC-HYG-102083-KO were isolated. Diagnostic PCR was carried out using the primer pair 36951-EXT-F/36951-INT-R which amplified a 1735 bp product specific to the Mycgr3G36951 wild type allele or a 2890 bp product amplified using 36951-EXT-F/HYGC-INT-R indicating replacement of the Mycgr3G36951 gene by the hygromycin resistance cassette. Similarly primer combination 72646-EXT-F/72646-INT-R and 102083-EXT-F/102083-INT-R amplified 1200 and 1400 bp products from wild type alleles of Mycgr3G72646 and Mycgr3G102083 respectively. The amplification of 1900 bp and 2100 bp PCR products using the primer pairs 72646-EXT-F/HYGC-INT-R and 102083-EXT-F/HYGC-INT-R confirmed the replacement of Mycgr3G72646 and Mycgr3G102083 by the hygromycin resistance cassette.

2.5. Sulfonylurea sensitivity screen

To titrate the optimal inhibitory concentration for selection with sulfonylurea, the Z. tritici wild type strain IPO323 and strain IPO323::SurR^1 were sub-cultured 10 times on non-selective YPD agar and then used for drug sensitivity screens. For each strain, yeast like cells were harvested and a 10 μl aliquot of cell suspension was spotted onto petri dishes containing BM agar amended with varying concentrations of sulfonylurea. After 24 h incubation at 25°C, the resistance of the colonies was scored relative to wild type strain HLS1000.
suspension (serially diluted to $10^6$, $10^5$, and $10^4$ cells/mL in sterile water) was spotted in triplicate on BM agar containing sulfonylurea concentrations ranging from 0 to 12 µg/mL. Plates were incubated at 18 °C for 6 days and images were acquired using a GBOX Chemi XX6 imaging system (Syngene).

2.6. Phenotypic characterization of the Z. tritici ku70 and ku80 null strains

Z. tritici strains HLS1000, HLS1001, HLS1002 and IPO323 were screened to identify phenotypes that may arise from the deletion of KU70 or KU80 genes. Mutagen sensitivity screens were carried out essentially as described by Ninomiya et al. (2004). Cells were spotted on SC agar amended with mutagens and plates were incubated at 18 °C for 6 days and images were acquired using a GBOX Chemi XX6 imaging system (Syngene, UK). For microscopic analysis, Z. tritici cells were harvested from cultures grown in SC, CDB and AMM and images were acquired using an Olympus IX81 spinning disc microscope (Olympus, Southend-on-Sea, UK). To determine the impact of Z. tritici KU70 and KU80 gene deletion on pathogenicity, whole plant infection assays (Motteram et al., 2009) were carried out. Briefly, the second leaf of 14 day-old wheat plants (susceptible cultivar Avalon) was inoculated with yeast like budding cells ($1 \times 10^7$ cells/ml in water containing 0.1% (v/v) Tween 20) of Z. tritici strains HLS1000, HLS1001, HLS1002 and the wild type strain IPO323. Mock infection was carried out using sterile water containing 0.1% (v/v) Tween20. Infected plants were maintained at 16:8 h day:night cycles at 18 °C and 85% relative humidity for 21 days. Visual inspections of infection were carried out at 14, 18 and 21 days after infection (DAI). Images were acquired with a Leica M205FA stereo microscope (Leica, Milton Keynes, UK) and Epson scanner (Epson, Hertfordshire, UK).

3. Results and discussion

3.1. Yeast recombinational cloning for vector construction

In a recent study, we have utilized yeast recombinational cloning to construct thirty two Z. tritici over-expression vectors (Sidhu et al., 2015). This technique circumvents resource-heavy conventional cloning which is often used to construct gene deletion vectors for Z. tritici. We therefore developed four A. tumefaciens ternary vectors for recombinational cloning in S. cerevisiae. The A. tumefaciens binary vector pC-HYG (Motteram et al., 2009) was modified by adding the S. cerevisiae 2µ origin of replication and

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Fig. 1. Z. tritici wild type strain IPO323 is hypersensitive to sulfonylurea (A) and genemically integrated IUV2<sup>Sur</sup> sulfonylurea resistance cassette confers resistance to the drug (B). (A) Z. tritici wild type strain IPO323 is hypersensitive to sulfonylurea (chlorimuron ethyl is the active ingredient) at low concentrations (2 µg/mL) while strains IPO323-Sur<sup>+</sup> (independent mutants containing a randomly integrated sulfonylurea resistance cassette) are drug resistant following 10 rounds of growth on non-selective media. Z. tritici cells (from $10^5$ to $10^6$ cells/ml) were spotted on BM agar supplemented with various sulfonylurea concentrations and plates were incubated at 18 °C for 6 days. The wild type strain IPO323 shows severe growth inhibition whereas the IPO323-Sur<sup>+</sup> mutant is resistant to sulfonylurea concentrations up to 12 µg/mL. (B) Agarose gel (1% w/v in TAE) image showing PCR confirmation of presence of the sulfonylurea resistance cassette in the genomic DNA isolated from 15 randomly selected IPO323-Sur<sup>+</sup> mutants (Lane 1–15). Amplification of a 558 bp PCR product confirms that the sulfonylurea resistance cassette is present in strains IPO323-Sur<sup>+</sup> 1–15, but absent in wild-type Z. tritici IPO323 (Lane 16/WT). The pC-SUR-YR vector Lane 17/V was used as positive control for amplification of the 558 bp product from the sulfonylurea resistance cassette.
the URA3 selection marker to give pC-HYG-YR (Fig. S1). The hygromycin resistance cassette in pC-HYG-YR was replaced with the 
ILV2\textsuperscript{SUR} sulfonylurea resistance cassette from \textit{M. oryzae} (Valent and Chumley, 1991), the bar gene for resistance against glufosinate ammonium (Kramer et al., 2009) or the nptII gene for resistance against geneticin (Bowler et al., 2010). The resulting sequence-verified vectors were designated pC-SUR-YR, pC-BAR-YR and pC-G418-YR, respectively (Fig. S1).

3.2. Sensitivity to sulfonylurea and functionality of the resistance cassette in \textit{Z. tritici}

To establish the sulfonylurea resistance cassette as a new positive selection marker for use in \textit{Z. tritici} it was necessary to confirm its sensitivity to sulfonylurea. \textit{Z. tritici} wild type strain IPO323 was highly sensitive to sulfonylurea and growth was completely inhibited at drug concentrations as low as 2 \( \mu \text{g/mL} \) (Fig. 1). However, for selection on cellophane discs, which are used for ATMT transformation of \textit{Z. tritici}, we observed that 10 \( \mu \text{g/mL} \) sulfonylurea was needed to eliminate background \textit{Z. tritici} IPO323 growth (data not shown).

To confirm the functionality of the sulfonylurea resistance cassette in \textit{Z. tritici}, the vector pC-SUR-YR was transformed into strain IPO323 and 15 sulfonylurea resistant transformants designated strain IPO323::Sur\textsuperscript{A} 1–15 were isolated after 14 days growth on BM agar. Diagnostic PCR was carried out on the genomic DNA to confirm the genomic integration of the resistance cassette and a 558 bp fragment specific to the sulfonylurea resistance cassette was amplified from strains IPO323::Sur\textsuperscript{A} 1–15 but the PCR product was absent in case of wild type \textit{Z. tritici} IPO323 (Fig. 1). Further, a single IPO323::Sur\textsuperscript{A} strain was sub-cultured for 10 rounds on non-selective medium and then spotted on BM agar containing 1–12 \( \mu \text{g/mL} \) sulfonylurea (Fig. 1). This strain retained drug resistance suggesting genomic integration of the sulfonylurea resistance cassette was mitotically stable even in absence of selection pressure. These data demonstrate that the sulfonylurea resistance cassette from \textit{M. oryzae} is a functional dominant selection marker in \textit{Z. tritici}.

3.3. Inactivation of the \textit{Z. tritici} \textit{ku}70 and \textit{ku}80 genes

To inactivate the predicted \textit{Z. tritici} \textit{ku}70 (Gene ID MycygrC3G85040) and \textit{ku}80 (Gene ID Mycygr3G400048) genes, the respective gene deletion vectors pC-G418-KU70-KO, pC-G418-KU80-KO and pC-SUR-KU80-KO were introduced into the wild type strain IPO323 via ATMT. Diagnostic PCR was used to test for replacement of \textit{ku}70 by the geneticin resistance cassette (Fig. 2A), using the primer pairs KU70-EXT-F/KU70-INT-R and KU70-EXT-F/G418\textsuperscript{3}-INT-REV. Out of 96 putative transformants tested, the amplification of a 2635 bp product in three suggested the replacement of \textit{ku}70 by the geneticin resistance cassette (Fig. 2C). However, amplification of a 928 bp product, using KU70-EXT-F/KU70-INT-R, in two out of these three putative \textit{ku}70 null strains also suggested the presence of a wild type \textit{ku}70 allele (Fig. 2C). The deletion of the \textit{ku}80 gene was confirmed by diagnostic PCR on another set of 96 putative mutants, harbouring the vector pC-G418-KU80-KO (Fig. 2B). A 3228 bp PCR product amplified using primers KU80-EXT-F/G418\textsuperscript{3}-INT-REV from the genomic DNA isolated from three mutants suggested the \textit{ku}80 gene had been replaced by the geneticin resistance cassette (Fig. 2D). However, amplification of a 1619 bp PCR product using primers KU80-EXT-F/KU80-INT-REV in one of these putative \textit{ku}80 null mutants suggested the presence of wild type \textit{ku}80 gene (Fig. 2D). Southern blot analysis confirmed that in the \textit{ku}70 and \textit{ku}80 null strains the target genes had been replaced by the geneticin resistance cassette without any ectopic integrations events (Fig. S2). These confirmed \textit{ku}70 and \textit{ku}80 null strains were designated \textit{Z. tritici} HLS1000 and HLS1001, respectively.

We also attempted to inactivate \textit{Z. tritici} \textit{ku}80 with pC-SUR-KU80-KO to test the utility of the sulfonylurea resistance...
cassette as a positive selection marker for gene deletion. The vector pC-SUR-KU80-KO was transformed into the wild type strain Z. tritici IPO323. Colony PCR was carried out, using a combination of three primers KU80-EXT-F/KU80-INT-R/SUR-INT-R (see Supplementary Table S1) to identify ku80 null strains (Fig. S3A). Out of 192 transformants that were screened, four showed a single 2490 bp amplicon, which confirmed that the deletion construct had integrated into the KU80 locus (Fig. S3B). Southern blot analysis showed that the DIG-dUTP labelled probe hybridized at a single 3792 bp band thus confirming gene deletion without ectopic integration of the deletion vector (Fig. S3C). The ku80 null strain constructed using the sulfonylurea resistance cassette was designated HLS1002. These experiments confirmed that the sulfonylurea resistance cassette can be used as a positive selection marker for gene deletion in Z. tritici.

3.4. Characterization of the Z. tritici ku70 and ku80 null strains

To test whether inactivation of the Z. tritici KU70 and KU80 gene in strains HLS1000 and HLS1001 respectively affected gene targeting frequency, we attempted to inactivate three genes (Table 1). Using ATMT each gene deletion construct was individually introduced into strains HLS1000, HLS1001 and wild type Z. tritici IPO323. Diagnostic PCR was carried out on 16 hygromycin resistant

<table>
<thead>
<tr>
<th>Gene name/ID</th>
<th>Size (bp)</th>
<th>Locus</th>
<th>HLS1000 (%)</th>
<th>HLS1001 (%)</th>
<th>IPO323 (%)</th>
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<td>Mycgr3G102083</td>
<td>2,058</td>
<td>chr_1:1020739-1022796</td>
<td>100</td>
<td>93.75</td>
<td>6.25</td>
</tr>
</tbody>
</table>

The sulfonylurea resistance cassette was designated HLS1002. These experiments confirmed that the sulfonylurea resistance cassette can be used as a positive selection marker for gene deletion in Z. tritici.

![Fig. 3](image)

**Z. tritici ku70 and ku80 null mutants are essentially wild type in respect of mutagen sensitivity, in vitro growth and pathogenicity.** (A) Z. tritici strains HLS1000 (ku70 null) and HLS1001 (ku80 null) are essentially wild-type in their response to methyl methanesulphonate (MMS), benomyl and hydroxyurea (HU), but exhibit mildly increased sensitivity to ultraviolet (UV) radiation as compared to the wild type strain IPO323. Serially diluted cells ($10^8$–$10^4$ cells/mL) were spotted on SC agar containing MMS (1 μl/mL), HU (1 mg/ml), benomyl (10 μg/mL) or exposed to UV radiation (350 J/m²). (B) Deletion of the Z. tritici KU70 and KU80 genes does not affect in vitro growth. Strains HLS1000, HLS1001 and IPO323 were grown in SC, CD or AMM for 72 h at 18°C and 120 RPM. Images were acquired in the bright field channel using an Olympus IX81 spinning disc microscope and Visiview imaging suite. Scale bar represents 10 μm. (C) Plant infection assay shows that strains HLS1000 and HLS1001 are pathogenic and induce typical symptoms (necrosis, chlorosis and formation of black/brown pycnidial lesions) of Z. tritici infection similar to the wild type strain IPO323. Whole leaves of wheat plants of susceptible cultivar Avalon were inoculated with $1 \times 10^2$ cells/mL and images were acquired 21 days after infection. (D) Quantitative analysis of infection (pycnidia per cm²) showed no significant difference in pathogenicity of strain HLS1000 ($p = 0.25$) and HLS1001 ($p = 0.55$) as compared to wild type strain IPO323. Pycnidia were counted manually and data was statistically analysed using analysis of variance (ANOVA).
transforms isolated from each background (HLS1000, HLS1001 and IPO323). The deletion frequency of the 13,961 bp long gene Mycgr3G36951 exceeded 90% in strains HLS1000 and HLS1001 as compared to 62.5% observed in the IPO323 background (Fig. S4 and Table 1). Similarly, the other two genes Mycgr3G72646 and Mycgr3G102083 were also targeted at an elevated frequency exceeding 85% in strains HLS1000 and HLS1001 as compared to less than 10% in the wild type strain (Table 1). These results reveal a dramatic increase in the gene targeting frequency as a result of inactivation of both the Z. tritici KU70 and KUB0 genes. This is in agreement with previously reported increased gene deletion frequencies that were achieved in an independently created Z. tritici ku70 null strain (Bowler et al., 2010). In addition, our ku70 null strain HLS1000 was also successfully used for high frequency targeted integration of gene overexpression constructs (Sidhu et al., 2015) and to replace the native promoter of the Z. tritici \(\beta\)-1,3-glucan synthase gene (Marchegiani et al., 2015).

Due to disruption of DNA repair, the NHEJ mutants can become hypersensitive to DNA damaging mutagens (Hoff et al., 2010). In line with observations in \(N.\) crassa (Ninomiya et al., 2004), the Z. tritici strains HLS1000 and HLS1001 showed mildly increased sensitivity to ultraviolet (UV) radiation but not to methyl methane sulphonate (MMS), hydroxyurea (HU) or benomyl (Fig. 3A). These compounds result in low mutant points but not in double strand breaks in DNA and therefore it is not surprising that both mutants are not hypersensitive to these mutagens. Strains HLS1000 and HLS1001 showed no obvious morphological growth defects in standard in vitro conditions (Fig. 3B). Similar to wild type Z. tritici IPO323, both strains exhibited yeast like cell growth in SC medium while pseudohyphal and hyphal growth was predominant when strains were grown in nutrient limiting CDM and AMM (Fig. 3B).

To be useful for in vivo analysis it is imperative that mutations that facilitate homologous recombination (such as deletion of KU70 and KUB0) and selectable markers (such as geneticin and sulfonylurea resistance cassettes) used for transformation of pathogenic fungi, do not detectably impact virulence during infection assays. To determine this for these strains wheat leaves (susceptible cultivar Avalon) were inoculated with Z. tritici HLS1000 strain (wild type progenitor strain HLS1000) and HLS1001. The similar progression of infection and specifically inspection of infection revealed no differences in the initiation of lesions at 18 DAI and necrosis at 21 DAI on the leaves infected with HLS1002 and the wild type progenitor strain IPO323. Visual chlorosis at 14 DAI, in appearance of typical black brown pycnidial lesions formed on leaves infected by strains HLS1000 and HLS1001 showed no obvious morphological growth defects in comparison to 6.25% observed in the IPO323 background (Fig. S3E).

In this study, we report the generation of new tools for construction of Z. tritici mutant strains. The confirmation of the functionality of the sulfonylurea resistance marker in Z. tritici increases the number of positive selection markers available for use in this fungus. Along with the ternary vectors offering yeast recombinational cloning, this represents a significant addition to the toolkit available for genetic manipulation of Z. tritici. ATMT is widely used to transform fungi and plants (Michielse et al., 2005) and therefore these vectors will also be useful for the wider fungal research community. The Z. tritici ku70 and kub0 null strains facilitate increased HR mediated gene targeting while maintaining wild type growth and pathogenicity in planta. Taken together, these tools complement currently available techniques and are a major step towards making large-scale gene functional analysis feasible in Z. tritici.

Acknowledgments

We thank Sreedhar Kilaru for technical assistance with the yeast recombinational cloning. This work was funded by a BBSRC BB/GR512557/1 and a BBSRC CASE studentship to KH (BB/J000793/1), supported by Syngenta UK.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2015.04.015.

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Appendix 5

A suite of Gateway® compatible ternary expression vectors for functional analysis in Zymoseptoria tritici

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1. Introduction

Septoria tritici leaf blotch (STB), a foliar disease of wheat (Triticum species), is caused by the ascomycete fungal pathogen Zymoseptoria tritici (Mycosphaerella graminicola) (Suffert et al., 2013). Every year STB outbreaks can reduce yields by 30–40% (Polley and Thomas, 1991), as such STB is globally regarded as an economically important cereal disease threatening food security (Goodwin et al., 2011). The potential threat posed by STB has driven research into understanding pathogen biology and molecular mechanisms underlying its mode of pathogenesis.

Functional genetic analysis in Z. tritici is predominantly restricted to targeted gene deletions using Agrobacterium tumefaciens mediated transformation (ATMT) (Bowler et al., 2010; Zwiets and De Waard, 2001; Motteram et al., 2009). Over the last decade, this invaluable technique has provided new insights into mechanisms underpinning growth, signaling, subversion of the host immune response, drug resistance and pathogenesis (Gohari et al., 2014; Stergiopoulos et al., 2003; Zwiets et al., 2003, 2007; Mehrabi et al., 2006a,b). However, the low homologous recombination frequency in Z. tritici is a major rate limiting factor in generating gene deletion mutants (Bowler et al., 2010). In addition essential genes which encode for potential drug targets, cannot be deleted (Oliveira-Garcia and Deising, 2013; Hu et al., 2007) and thus the function of their encoded proteins cannot be studied using gene deletion. Therefore, it is vital to develop new tools and establish alternative strategies for functional genomics in Z. tritici.

In model organisms such as Saccharomyces cerevisiae (Stevenson et al., 2001; Chua et al., 2006) and Schizosaccharomyces pombe (Matsuyama et al., 2006) plus the pathogenic fungus Candida albicans (Chauvel et al., 2012) gene overexpression is widely used for functional characterization. Gene overexpression in these species was enabled by the development of high throughput functional analysis tools, for example expression vectors (Alberti et al., 2007; Chauvel et al., 2012; Nagels Durand et al., 2012) and genome-wide open reading frame (ORF) libraries (Chauvel et al., 2012; Gelperin et al., 2005; Matsuyama et al., 2006). In addition to gene overexpression, these tools allow tagging of ORFs with protein affinity tags or fluorescent proteins for tandem-affinity protein purification and subcellular localization studies (Alberti et al., 2007). Expression vectors and ORFeomes made it possible to conduct large scale cell biological studies which involved fluorescent protein tagging of 6029 and 4431 ORFs in the model organisms S. cerevisiae and S. pombe, respectively (Huh et al., 2003; Matsuyama et al., 2006). These tools have been pivotal in functional characterization by revealing novel biological information on subcellular protein localization,
transcriptional co-regulation as well as interactions between cytoskeletal organelles (Huh et al., 2003; Matsuyama et al., 2006). However, due to the lack of such tools in *Z. tritici*, overexpression and protein fusion has not been used on a large scale for gene characterization.

To address this bottleneck, we have constructed a suite of 32 expression vectors for overexpression and protein fusion to enhanced green fluorescent protein (EGFP) in *Z. tritici*. All vectors were constructed in the *Escherichia coli* [A. tumefaciens] *S. cerevisiae* ternary vector pC-HYG-YR (Sidhu et al., 2015). The vectors can be propagated in *E. coli*, facilitate cloning by recombineering in *S. cerevisiae* (Collopy et al., 2010) and allow ATMT of expression constructs into *Z. tritici*. All vectors take advantage of the Gateway® recombinant technology (Life Technologies Ltd, Paisley, UK) and offer a choice of three constitutive and one inducible *Z. tritici* promoter, four positive selection markers, and come with, or without, C-terminal EGFP fluorescent tag. Here we describe construction of these vectors and show their potential application for gene overexpression and protein fusion in *Z. tritici*.

2. Materials and methods

2.1. Strains and growth conditions

All chemicals and reagents used in this study were purchased from Sigma–Aldrich (Dorset, UK), unless otherwise stated. The *Z. tritici* ku70 null strain HLS1000 (*Aku70:G418*) (Sidhu et al., 2015) was used as the recipient strain for overexpression vector transformation. Yeast Peptone Dextrose agar (YPD agar, per litre: 10 g yeast extract, 20 g peptone, 20 g dextrose and 2 g agar) was used for culturing *Z. tritici* strains. Induction medium (IM agar) was used for ATMT. *Z. tritici* strain BY4741 (Ku70 null strain HLS1000), the *Ku70* lesion in the *Z. tritici* strain, the *Hyg* cassette (for selection in *Z. tritici*), the *Z. tritici* promoter pZtTEF (promoter of the putative transcription elongation factor encoding gene *Mycgr3G29705*, to drive ORF expression), an *attR1-ccdB-attrR2* Gateway® cassette (to enable ORF cloning from Gateway entry vectors) and either a C-terminal stop codon or EGFP gene (to allow over-expression of native or tagged proteins). To achieve this *pC-HYG-YR* was double digested with *AscI* and *BstEII* restriction endonucleases and the resulting fragment was ligated into *E. coli* TOP10 ccdB® (Life Technologies Ltd) was used to propagate plasmids containing the Gateway® cassette, all other plasmids were propagated in *E. coli* strain DH5α. Bacterial cultures were grown at 37 °C in Luria–Bertani (LB) (Formedium™, Hunstanton, UK) media supplemented with 100 µg/mL kanamycin. *A. tumefaciens* strain EHA105 was used for the ATMT of *Z. tritici*; cultures were grown at 30 °C in LB or IM broth containing 100 µg/mL kanamycin and 100 µg/mL rifampicin.

2.2. Nucleic acid manipulations

Standard protocols (Sambrook and Russell, 2001) were followed for all DNA manipulations, unless otherwise stated. Phusion® high fidelity polymerase (New England Biolabs (NEB), Herts, UK) was used in PCR amplification for cloning and GoTaq® flexi DNA polymerase (Promega, Southampton, UK) was used for colony PCR. All primers used in this study are shown in supplementary Table 1. Restriction endonucleases were purchased from NEB. DNA fragments were purified from agarose gel using silicon dioxide and sodium iodide (Li et al., 2010). The Sanger sequencing service provided by Eurofins Genomics (Ebersberg, Germany) was used to sequence verify all plasmids. A standard phenol chloroform extraction protocol (Mortemar et al., 2009) and Fastprep® cell disruptor (Fisher Scientific, Loughborough, UK) were used to extract *Z. tritici* genomic DNA. A Qiagen spin miniprep kit (Qiagen, Manchester, UK) was used to isolate plasmids from *E. coli*.

Recombinant plasmids were rescued from *S. cerevisiae* using the lyticase and Qiaprep protocol (Singh and Weil, 2002).

2.3. Construction of expression vectors

Using the ternary vector *pC-HYG-YR* (Sidhu et al., 2015), we engineered two vectors, which contained *Ku70* left and right flanking regions (for targeting to the *Z. tritici* *Ku70* locus) a functional *Ku70* gene (for complementation of the *ku70* lesion in the *Z. tritici* strain HLS1000), the *Hyg* cassette (for selection in *Z. tritici*), the *Z. tritici* promoter pZtTEF (promoter of the putative transcription elongation factor encoding gene *Mycgr3G29705*, to drive ORF expression), an *attR1-ccdB-attrR2* Gateway® cassette (to enable ORF cloning from Gateway entry vectors) and either a C-terminal stop codon or EGFP gene (to allow over-expression of native or tagged proteins). To achieve this *pC-HYG-YR* was double digested with *AscI* and *BstEII* restriction endonucleases and the 9153 bp backbone was gel purified. PCR was used to amplify all other components (see supplementary Table 2) and these were transformed together with the double digested *pC-HYG-YR* backbone into *S. cerevisiae* BY4741 (Sidhu et al., 2015). Plasmids were recovered from *S. cerevisiae* uracil prototrophs and sequenced and designated pYSKH3. Similarly an appropriately recombined, sequence verified vector with a C-terminal EGFP protein fusion was selected and designated pYSKH15; Fig. 1 shows a schematic of the constructed vectors. The resulting Gateway® compatible expression vectors pYSKH3 and pYSKH15 were used as backbones to construct a further 30 vectors (see supplementary Table 3) by replacing the *Hyg* resistance cassette with one of three other resistance cassettes bar², *G418*® or *Sur*, and/or the *pZTEF* promoter with one of three other *Z. tritici* promoters (Fig. 1); *pZGAPDH* (promoter of the putative glyceraldehydes-3-phosphate dehydrogenase encoding gene *Mycgr3G99044*), *pZATUB* (promoter of the putative α-tubulin encoding gene *Mycgr3G76019*) and *pZNA1* (promoter of the putative nitrate reductase encoding gene *Mycgr3G111003*). To construct the vectors pYSKH3 and pYSKH15 were double digested with *AatI* and *MfeI* restriction endonucleases and the resulting 16,020 bp and 16,723 bp backbones respectively, were individually gel purified. The *Z. tritici* promoters and resistance markers were PCR amplified (see supplementary Tables 1 and 2) and assembled using yeast recombineering as described above. Recombinant plasmids were isolated from uracil prototrophic *S. cerevisiae* and propagated in *E. coli* TOP10 cells. Appropriate recombination of cloned fragments was confirmed by Sanger sequencing and recombined expression vectors were designated pYSKH 1-32 (see supplementary Table 3).
transformants per strain were sub cultured for two further rounds of selection on YPD agar containing hygromycin (200 μg/mL) and plates were maintained 18 °C for 6 days.

2.6. PCR confirmation of in-locus integration of the construct

Genomic DNA was extracted from yeast like cells of putative Z. tritici over-expressing strains and the parental strain HLS1000 using phenol chloroform extraction (Mottaram et al., 2009). To confirm integration of the overexpression plasmids at the Z. tritici ku70 locus, diagnostic PCR was carried out using the primer pair pYSKH-INT-FWD and KU70-EXT-REV which amplify a 1510 bp product only if the expression construct has integrated into the mutated Ku70 locus in the ku70 null strain HLS1000 (Fig. 2A and B).

2.7. Microscopic analysis and quantification of fluorescence

Z. tritici cultures were grown in YPD broth for 72 h at 18 °C at 180 RPM and 1 mL aliquots were centrifuged at 13,000 RPM for 1 min and cell pellets were washed with sterile water. Microscopic analysis for quantification of fluorescence was carried out as described by Bielska et al. (2014). Briefly, 3 μL cells were spotted on 2% (w/v) agarose on a glass slide. The 488 nm emission laser was used at 20% intensity to excite EGFP protein and images were acquired at 200 ms exposure using an Olympus IX81 spinning disc microscope (Olympus, Southend-on-Sea, UK) and Visiview imaging suite (Visitron Systems GmbH, Puchheim, Germany). Imagej version 1.48 (http://imagej.nih.gov/ij/) was used to measure the cytoplasmic and background fluorescence intensities. Each experiment was repeated twice and data from 100 individual measurements (50 per biological replicate/strain) were pooled for analysis.

3. Results and discussion

3.1. Overview of the Gateway® cloning and destination expression vectors

We constructed a suite of 32 Gateway® expression vectors (designated pYSKH 1-32; see Table S2) in the A. tumefaciens backbone plasmid pC-HYG-YR (Sidhu et al., 2015) using recombineering in S. cerevisiae (Collopny et al., 2010). In each expression vector (Fig. 1), the transfer DNA (TDNA) region between left (LB) and right (RB) borders contains one of four resistance cassettes barR, G418R, HygR and SurR (Sidhu et al., 2015) and one of four Z. tritici promoters (pZtNIA, pZtGAPDH, pZtTEF and pZtATUB) that control expression of the cloned ORF (see supplementary Table 2). These include three constitutive promoters pZtATUB, pZtGAPDH and pZtTEF of different strength; and a fourth inducible promoter pZtNIA1 which is induced by nitrate and repressed by glutamic acid or other nitrogen sources (Marchegiani et al., 2015). Of the 32 vectors, 16 contain a direct C-terminal stop codon to overexpress native proteins, and the other 16 vectors contain a C-terminal EGFP fluorescent tag that allows in-frame cloning of the ORF with the tag separated by a short linker sequence. In S. cerevisiae this short linker sequence between the ORF and the tag does not interfere with protein targeting and folding (Alberti et al., 2007). However, it is important to stress that in some cases the function of a protein may be adversely affected as a result of the linker sequence (Alberti et al., 2007). In order to exploit the increased homologous recombination frequency of ku70 null strains we have used Z. tritici HLS1000 for our overexpression studies. We have included both a functional Ku70 gene and Ku70 flanking sequences in the pYSKH plasmids to facilitate the targeting of expression vectors into the mutated ku70 locus in Z. tritici HLS1000, while also complementing the Ku70 gene at the same time. We have therefore
created a set of Gateway compatible ternary vectors that have one of four positive selection cassettes, one of four promoters, have or lack a C-terminal EFGP and are targeted to the same ku70 soft-landing site in *Z. tritici* HLS1000.

### 3.2. Confirmation of the functionality of destination vectors in *Z. tritici*

To confirm that the pYSKH34/35/36/46/47 overexpression cassettes integrated into the ku70 soft-landing site in *Z. tritici* HLS1000 we carried out diagnostic PCR on the genomic DNA isolated from putative hygromycin resistant transformants from each transformation. If the overexpression cassette integrates as expected a 1500 bp PCR product will be amplified (Fig. 2A). All hygromycin resistant transformants of *Z. tritici* strain HLS1004 (containing pYSKH34) amplified the expected 1500 bp band, this was not seen in the parental strain HLS1000 (Fig. 2B). Similar results were seen with all other overexpression cassettes. One individual transformant was then selected and designated as follows *Z. tritici* HLS1004 (*Δku70::G418*::*Ku70:ptrpC:Hyg<sup>R</sup>:pZTTEF-EFGP), HLS1005 (*Δku70::G418*::*Ku70:ptrpC:Hyg<sup>R</sup>:pZTTEF-EFGP), HLS1006 (*Δku70::G418*::*Ku70:ptrpC:Hyg<sup>R</sup>:pZATUB-EFGP), HLS1007 (*Δku70::G418*::*Ku70:ptrpC:Hyg<sup>R</sup>:pZTTEF::Mycgr3G103593-EFGP) and HLS1008 (*Δku70::G418*::*Ku70:ptrpC:Hyg<sup>R</sup>:pZTTEF::Mycgr3G42164-EFGP). This confirms that the 1000 bp and 800 bp ku70 left and right flanks present in each expression vector (Figs. 1 and 2A) facilitates efficient targeting of the expression construct into the ku70 soft-landing site.

Following confirmation of their targeting to *ku70* we sought to determine the functionality of the *Z. tritici* constitutive promoters in these overexpression vectors by analyzing their ability to drive the expression of the heterologous fluorescent reporter gene EFGP. Hence we quantified the fluorescence intensity of the EFGP protein in yeast like cells of *Z. tritici* strains HLS1004, HLS1005, and HLS1006 whereas only very weak fluorescence was observed in HLS1000 strains (Fig. 2C).

The quantitative analysis
of the cytoplasmic fluorescence intensity revealed that p2ITEF was the strongest Z. tritici promoter followed by p2GAPODH and p2ATUB (Fig. 2D). These data confirm that the Z. tritici promoters can drive expression of genes cloned into the Gateway® site of pYSKH vectors. To confirm that the vectors can also be used for analysis of protein fusions, we constructed Z. tritici strains HLS1007 and HLS1008, which contained Mycgr3G42164-EGFP and Mycgr3G103593-EGFP fusions respectively, both under the control of the p2ITEF promoter. Mycgr3G103593 encodes a 154 peptide which contains a Cu–Zn superoxide dismutase domain and has 70% identity to SOD1 (superoxide dismutase 1) in S. cerevisiae which plays a role in the oxidative stress response and localizes to both cytoplasm and nucleus (Tsang et al., 2014). BLASTP searches revealed that Z. tritici Mycgr3G42164 encodes a 301 amino acid peptide with 48%, 35% and 29% identity to proteins encoded by the genes AspF2 (AFUA_G09580) in Aspergillus fumigatus, ZPS1 (YOL154W) in S. cerevisiae and PRA1 in C. albicans. The AspF2, PRA1 and ZPS1 genes are induced under zinc depletion and facilitate zinc uptake in A. fumigatus, C. albicans and S. cerevisiae (Cituilo et al., 2012; Amich et al., 2010). BLASTP and Gene Ontology analysis suggested that the Mycgr3G42164 protein contains an M35 superfamily domain for metalloproteases and may be associated to vacuoles (GO:0000324) and/or cell wall (GO:0000277). Visual analysis revealed clear differences in the subcellular localization of the fusion protein in each strain. The Mycgr3G103593-EGFP fusion protein displayed strong cytoplasmic abundance whereas the Mycgr3G42164-EGFP fusion appeared to be restricted to certain subcellular organelles (Fig. 2D and E). These results show that the vectors can be used to tag and localize proteins in Z. tritici. Taken together, these results show that the three constitutive promoters namely p2ITEF, p2GAPODH and p2ATUB are functional in Z. tritici. Similarly, q-RT PCR and analysis of fluorescence intensity revealed the nitrous resistant promoter p2UNA1 was induced in the presence of nitrate and repressed upon exogenous application of glutamic acid, glutamine and ammonium (Marchegiani et al., 2015). These results confirm that all four promoters can be used for gene overexpression in Z. tritici. In the model organism S. cerevisiae a large phenotypic screen revealed that overexpression of a subset of 371 ORFs lead to deleterious growth defects and lethality (Gelperin et al., 2005). This provides a clear example of how gene overexpression will underpin the mode of pathogenesis deployed by Z. tritici. 

4. Conclusions

In conclusion, we have reported a suite of 32 ternary expression vectors potentially useful for gene characterization in Z. tritici using overexpression and tagging. By complementing the existing tools such as the mK70 null strain, these vectors will facilitate rapid construction of Z. tritici overexpressing or tagged strains and will enable the Z. tritici research community to exploit the full benefits of the Z. tritici ORFeome (Y.K. Chaudhari, Y.S. Sidhu, T. C. Cairns, D.J. Studholme and K. Haynes unpublished). The vectors will serve as a robust platform for gene characterization by high throughput in vivo screening on various biotic and abiotic stress conditions encountered by the pathogen during invasion of the host.

Acknowledgments

We thank Eileen Scott and Sreedor Kilaru for advice and assistance with vector construction. This work was funded by a BBBSRC BBR grant (BB/I025956/1) to KH and collaborators and a BBSRC CASE studentship (BB/j500793/1), supported by Syngenta UK.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.fgb.2015.03.021.

References


Appendix 6

Conditional gene expression and promoter replacement in Zymoseptoria tritici using fungal nitrate reductase promoters

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A R T I C L E   I N F O

Article history:
Received 4 February 2015
Revised 23 April 2015
Accepted 27 April 2015

Keywords:
Mycosphaerella
Nitrate reductase
Conditional promoter
Promoter exchange
Essential gene
Zymoseptoria

A B S T R A C T

Studying essential genes in haploid fungi requires specific tools. Conditional promoter replacement (CPR) is an efficient method for testing gene essentiality. However, this tool requires promoters that can be strongly down-regulated. To this end, we tested the nitrate reductase promoters of Magnaporthe oryzae (pMO NIA1) and Zymoseptoria tritici (pZtNIA1) for their conditional expression in Z. tritici. Expression of EGFP driven by pMO NIA1 or pZtNIA1 was induced on nitrate and down-regulated on glutamate (10-fold less than nitrate). Levels of differential expression were similar for both promoters, demonstrating that the Z. tritici nitrogen regulatory network functions with a heterologous promoter similarly to a native promoter.

To establish CPR, the promoter of Z. tritici BGS1, encoding a β-1,3-glucan synthase, was replaced by pZtNIA1 using targeted sequence replacement. Growth of pZtNIA1::BGS1 CPR transformants was strongly reduced in conditions repressing pZtNIA1, while their growth was similar to wild type in conditions inducing pZtNIA1. This differential phenotype demonstrates that BGS1 is important for growth in Z. tritici. In addition, in inducing conditions, pZtNIA1::BGS1 CPR transformants were hyper-sensitive to Calcofluor white, a cell wall disorganizing agent. Nitrate reductase promoters are therefore suitable for conditional promoter replacement in Z. tritici. This tool is a major step toward identifying novel fungicide targets.

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1. Introduction

Zymoseptoria tritici (syn. Mycosphaerella graminicola) is the causal agent of Septoria tritici leaf blotch (STB), a major foliar disease of wheat (Te beest et al., 2013). Z. tritici is characterized by a high genetic diversity and rapid adaptation to host plants and environment (Stukenbrock, 2013). Recent developments in the genomics of Z. tritici have significantly increased our understanding of this plant-fungal interaction (Perez-Nadales et al., 2014). However, some molecular tools such as conditional promoters are not yet available in Z. tritici. Such promoters are needed for identifying essential genes using conditional promoter replacement (CPR) (Lamb et al., 2013; Delic et al., 2013) or for heterologous protein production (Helmschrott et al., 2013). In filamentous fungi, expression of genes involved in nitrate (NO3−) assimilation is tightly controlled by exogenous nitrogen sources (Wong et al., 2008). Promoters of such genes are good candidates for constructing conditional expression vectors. Genes involved in NO3 utilization have been extensively studied in Aspergillus nidulans (Wong et al., 2008). This metabolic pathway involves nine genes encoding biosynthetic enzymes or transporters (Wong et al., 2008; Bolton and Thomma, 2008), i.e. two nitrate transporters (NtrA, NtrB), a nitrate reductase (NiaD), a nitrite reductase (NiaI), and 5 enzymes involved in biosynthesis of molybdobacte cofactor (Probst et al., 2014). In Aspergillus species, niaD, niaI and cnrB/ntrB are organized as a cluster (Sirot and Hibbett, 2007), and a bidirectional promoter controls the expression of niaD and niaI (Chang et al., 1996). Expression of NO3 utilization genes is positively regulated by two transcription factors (TF) acting cooperatively, a nitrate-specific zinc finger TF NiaR and a nitrogen-status sensing GATA TF AreA.

We searched for a nitrate reductase encoding gene (NIA1) in Z. tritici genome and studied its expression during growth on different nitrogen sources using qRT-PCR and promoter-EGFP fusions. We also tested a heterologous fungal nitrate reductase promoter from Magnaporthe oryzae using similar techniques. We used the promoter of Z. tritici NIA1 (pZtNIA1) to construct promoter replacement (CPR) vectors for studying essential genes in Z. tritici. Fungal β-1,3-glucan synthases are involved in the biosynthesis of cell wall glucans. The catalytic sub-unit is encoded by a single gene (BGS1) described as essential for growth in Aspergillus fumigatus,
Coccidioides posadasi and Colletotrichum graminicola (Firon and d’Enfert, 2002; Kelner et al., 2005; Oliveira-Garcia and Deising, 2013). Replacement of the native Z. tritici BGS1 promoter with pZtNIA1 and phenotypic screening of resulting transformants sug-
gested that BGS1 is important for growth in Z. tritici and is involved in tolerance to cell wall stress induced by Calcofluor White (CFW).

2. Material and methods

2.1. Culture media and strains

Chemicals and reagents were purchased from Sigma–Aldrich (Manchester, UK and Saint-Quentin, France). Z. tritici strain IPO323 (Kema and van Silfhout, 1997) was used as wild type (WT). CPR con-
structs were transformed into Z. tritici strain HLS1000 (IP0323 Δka70::G4188) which offers increased frequency of homologous recombination due to inactivation of KU70 (Sidhu et al., 2015a). Z. tritici strains were grown at either 18 °C or 25 °C for 3–6 days on Yeast Peptone Dextrose (YPD, Bowler et al., 2010), Potato Dextrose (PD, Acumedia-Neogen, Lansing, Michigan, USA) and Minimal Medium (MM-Zt + No3. Supplementary Table 1). NaN03 was replaced by 23 mM ammonium tartrate (MM-Zt + NaH, glutamate (MM-Zt + Glu) or glutamine (MM-Zt + Gln) for ATMT, Agrobacterium tumefaciens cultures were grown in Induction Medium (IM) (Bowler et al., 2010). S. cerevisiae strain BY4741 (MATa, his3, leu2, met15, ura3) was grown in YPD at 30 °C and 180 RPM for 12 h and used for transformation (Gietz and Woods, 2002). Uracil prototrophic transformants were selected on Synthetic Complete without URA, SC-URA, for 1: 6.9 g yeast nitro-
gen base with ammonium sulfate without amino acids (Formedium™, Hunstanton, UK), 790 mg complete amino acid sup-

2.2. Construction of ATMT binary vectors

A 2903 bp EcoRI-XbaI pMoNIA1::GFP fragment was excised from plasmid pBS4 (kindly donated by Marie-Josèphe Gagey, UMR MAP, Lyon, France) and cloned into pBTH2 vector (Motteram et al., 2009). Means and standard deviations were

2.3. ATMT of Z. tritici and transformant characterization

pELMA1, pYSHK51 and pYSHK101 were introduced into A. tumefaciens AGL1 or EHA105 strains by electroporation. ATMT of

2.4. RNA isolation and quantitative RT–PCR analysis

Total RNA was extracted from 5 μg of freeze dried fungal mycelia

using the RNeasy Plant Mini Kit (QIAGEN, Courtaboeuf, France) and CFX96 Real-Time System

(Supplementary Table 2) which amplify a 3790 bp fragment specific of WT ZtBGS1 promoter and a 5580 bp specific for pZtNIA1 CPR.

2.5. Quantitative fluorescence assay

Z. tritici transformants ZtpELMA1_1-3 and HLS1009_1-3 were grown in MM-Zt supplemented with NO3 or Glutamate (0, 10, 20, 30, 40 and 50 mM) for 3 days at 18 °C and 180 RPM. Quantification of fluorescence was carried out as described by Bielska et al. (2014) and Schuster et al. (2011). Briefly, 3 μl of cell suspension was spotted on water agarose (2% w/v) coated on a glass slide, and immediately exposed to solid-state laser (488 nm/50 mW) (Visitron Systems, Munich, Germany) for EGFP excitation. Images were acquired in GFP channel using 100× oil objective at 200 ms exposure using Olympus IX81 motorized inverted spinning disc microscope (Olympus, Hamburg, Germany).
Germany) and Visview imaging suite (Vistron Systems). Images were analyzed using Image J [version 1.48 (http://imagej.nih.gov/ij/)] to calculate cytoplasmic fluorescence signal intensity. For this, signal intensity was measured from a randomly selected cytoplasmic region of interest within the cell and background intensity was measured by moving the region of interest outside the cell where fluorescence was not affected by other fluorescent signals in the vicinity. Background signal intensity was subtracted from cytoplasmic signal intensity to calculate cytoplasmic fluorescence signal intensity. Average cytoplasmic fluorescence signal intensity for each condition was calculated from 100 individual measurements of intensity (50 per biological replicate) for each strain.

2.6. Phenotypic analysis of CPR transformants

HLS1011_1–3 transformants (pZtNIA1::BGS1 CPR) were grown on MM-Zt containing NO3 or Glu. Serially diluted yeast-like cells of HLS1011 and "WT" HLS1000 (ΔnuT:G418) were spotted on these media for 6 days at 18 °C. Serially diluted cells of both transformants were also spotted on MM-Zt + NO3 agar with 100 mg/l CEW or Congo Red (CR) and incubated for 6 days at 18 °C. Growth was qualitatively scored for each cell dilution. Images were acquired using G:BOX Chemi XX6 imaging system (Syngene, Cambridge, UK).

3. Results

3.1. Identification of the Z. tritici nitrate reductase gene NIA1

BLASTP search of the Z. tritici genome database (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html) using A. nidulans NiaD protein sequence (XP_656610.1) as query identified a single hit on chromosome 10 (XP_003849160.1, 0.0 E-value) corresponding to Z. tritici NIA1. Alignment of Z. tritici NIA1 with known fungal nitrate reductase protein sequences and construction of the corresponding phylogenetic tree (Supplementary Figs. 1A and 1B) revealed that Z. tritici NIA1 is orthologous to niaD. The 1172 bp intergenic region between the Z. tritici NIA1 and nitrate reductase-encoding gene NII1 (XP_003848835.1) contains two putative binding sites (5’ CTCGGHGG 3’, Punt et al., 1995) for a Nir2 (XP_003852092.1, ortholog of nirA) like zinc finger transcription factor and thirteen putative GATA binding sites for a Nir2 (XP_003852092.1, ortholog of areA) like transcription factor (Punt et al., 1995) (Supplementary Fig. 2). The Z. tritici NII1–NIA1 intergenic region is likely a bidirectional promoter, since NII1 is transcribed in the opposite direction to NIA1, and Nir1 and Nir2 TF putative binding sites are found in this region. The Z. tritici nitrate transporter encoding genes NTR1 (XP_003847717.1) and NTR2 (XP_003848428.1) are not co-located on chromosome 10 with the NII1–NIA1 cluster. This organization differs from Aspergillus spp. in which the nitrate transporter encoding gene ntrR is located adjacent to niaD and niaA (Slot and Hibbett, 2007). As observed in Z. tritici, Capnodiales such as Dothistroma septosporum, Cladosporium fulvum, Septoria muscula, and Mycosphaerella fijiensis have a bidirectional a NII1–NIA1 promoter and unlinked transporters. Other Dothideomycetes such as Cochliobolus heterostrophus, Leptosphaeria maculans or Parastagonospora nodorum have a different NIA1 locus organization (no NII1–NIA1 cluster, no divergent promoter, Slot and Hibbett, 2007).

3.2. Conditional expression in Z. tritici using different nitrate reductase promoters

M. oryzae (pMoNIA1) and Z. tritici (pZtNIA1) nitrate reductase gene promoters were tested in Z. tritici as transcriptional EGFP fusions. Plasmids pELMA1 and pYSKH51 carrying transcriptional fusions between EGFP and pMoNIA1 or pZtNIA1 respectively, were introduced into WT Z. tritici. On MM-Zt + NO3 (nitrate), transformants HLS1009 (1) (pIP323 pZtNIA1::EGFP) and ZtpELMA1_1 (pIP323 pMoNIA1::EGFP) displayed a strong cytoplasmic EGFP fluorescence (Fig. 1A). The intensity of fluorescence reached similar values for both transformants. The intensity of fluorescence of HLS1009 and ZtpELMA1 on nitrate did not vary according to concentrations tested (10, 20, 30, 40 and 50 mM). On MM-Zt + Glu (glutamate), a strong reduction in EGFP fluorescence (10-fold compared to nitrate) was observed for both transformants (Fig. 1B). Ectopic pMoNIA1::EGFP and native Z. tritici NIA1 expression levels were also quantified using qRT-PCR (Fig. 2C). The expression of NIA1 in WT Z. tritici was 40 and 7-fold higher in MM-Zt + NO3 than in YPD and PD, respectively (Fig. 2C). Replacement of nitrate in MM by glutamate (MM-Zt + Glu), ammonium (MM-Zt + NH4) or glutamine (MM-Zt + Gln) reduced Z. tritici NIA1 expression by 9, 7 and 5-fold, respectively as compared to NO3 (Fig. 2C). Expression of EGFP driven by pMoNIA1 in ZtpELMA1_1 was also quantified by qRT-PCR (Fig. 2C). On MM-Zt containing Glu, NH4 and Gln the expression of EGFP was down regulated by 18, 17 and 4-fold respectively as compared to NO3 in ZtpELMA1_1. These results showed that pMoNIA1 is regulated in similar way as pZtNIA1 in Z. tritici. Overall, expression of EGFP driven by pZtNIA1 and pMoNIA1 was high in the presence of NO3 as sole nitrogen source, while it was strongly down regulated in the presence of reduced nitrogen sources (Glu, NH4) for both promoters.

3.3. Conditional promoter replacement using the Z. tritici nitrate reductase, NIA1, promoter

BLASTP search of the Z. tritici genome database (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html/) using A. fumigatus β-1,3-glucan synthase protein sequence (Hu et al., 2007) identified a single BGS1 hit (Mycgr3G101655; XM_003848403; 80% amino acid identity). We constructed a pZtNIA1::BGS1 CPR vector (using pYSKH101 as backbone, Fig. 2A) containing the NIA1 Z. tritici promoter (1 kb) associated with a 1.2 kb BGS1 left flank, starting at 500 bp upstream of its start codon, and a 3 kb BGS1 right flank corresponding to BGS1 first exons in frame with pZtNIA1. When introduced by ATMT into “WT” HLS1000 (ΔnuT:G418), pYSKH101 allowed the replacement of the native Z. tritici BGS1 promoter with pZtNIA1 (Fig. 2B). Diagnostic PCR using Z. tritici BGS1 promoter spanning primers BGS1-EXT-FWD and BGS1-INT-REV (Fig. 2B) amplified a 5580 bp fragment in all pZtNIA1::BGS1 CPR transformants tested (HLS1011 1-3) and a 3780 bp fragment in “WT” HLS1000 (Fig. 2C). These results confirmed that the native Z. tritici BGS1 promoter was replaced by pZtNIA1 in HLS1011 transformants with a high efficiency (100%).

The three pZtNIA1::BGS1 CPR transformants (HLS1011 1-3) and “WT” were grown on MM-Zt + NO3 or MM-Zt + Glu for 6 days at 18 °C. In NIA1 inducing conditions (MMZt + NO3), pZtNIA1::BGS1 CPR transformants displayed a similar growth as “WT” HLS1000 at all inoculum concentrations tested (Fig. 2D). In NIA1 repressing conditions (MM-Zt + Glu), pZtNIA1::BGS1 CPR transformants (HLS1011 1-3) showed a similar growth as “WT” HLS1000 at high inoculum (10^5 cells/ml), but a strong reduction in growth (faint or no colony) at lower inoculum (10^4 cells/ml) (Fig. 2D) compared to “WT” HLS1000 (same growth at low and high inoculum). The leaky phenotype of pZtNIA1::BGS1 CPR transformants could be due to the residual transcription driven by pZtNIA1 in repressing conditions as observed in our qRT-PCR experiments (Fig. 1B and C). CFW and CR are toxic to fungi through their ability to bind to cell wall polysaccharides and destabilize this structure (Ram and Klis, 2006). In NIA1 inducing conditions (MM-Zt + NO3), pZtNIA1::BGS1 CPR
transformants (HLS1011 1-3) displayed a strong hyper-sensitivity to 100 mg/l CFW and CR (no growth at all inoculum concentrations; Fig. 2E) compared to “WT” HLS100 that is still able to grow at these CFW and CR concentrations. These results
suggest that *Z. tritici* BGS1 is important for growth and sensitivity to cell wall stress.

4. Discussion and conclusion

In this study, we showed that pZtNIA1 and pMoNIA1 promoters are useful to control the expression of candidate genes in *Z. tritici*. Both promoters lead to a high level of expression in the presence of nitrate, while in the presence of glutamate, the expression is down-regulated by 18-fold according to qRT-PCR. Both promoters lead to a high level of expression in the presence of nitrogen sources.

We have established a strategy for CPR in *Z. tritici* using pZtNIA1. The native promoter of the *Z. tritici* β-1,3-glucan synthase encoding gene, BGS1, was successfully replaced with the pZtNIA1 promoter using a gene replacement vector and the *ku70* "WT" strain *HLS1000* to facilitate targeted gene replacement. Growth of pZtNIA1::BGS1 CPR transformants was similar to "WT" in inducing conditions (nitrate), but strongly reduced in repressing conditions (glutamate) at low inoculum. This result suggests that *Z. tritici* BGS1 is important for growth. BGS1 was already described as essential for growth in different fungi. In *A. fumigatus*, *Firon and d’Enfert* (2002) were unable to obtain viable haploid *bsg1* (bsg1) deletion mutants from heterozygous diploid transformants carrying *bsg1* deletion and WT alleles.

![Fig. 2. Replacement of the *Z. tritici* BGS1 promoter with pZtNIA1. (A) pC-HYG-pZtNIA1-YP vector. This vector contains a Hyg^R cassette and a pZtNIA1 located between left (LB) and right (RB) T-DNA borders. This vector carries a 2µ replication origin and URA3 for propagation and selection in *S. cerevisiae*; and Kan^R for selection in *A. tumefaciens* and *E. coli*. (B) Strategy for the construction of *Z. tritici* pZtNIA1::pZtBGS1 CPR transformants. Targeted replacement of the native *Z. tritici* BGS1 promoter with pZtNIA1 was performed in a Ku70 null mutant background (HLS1000). (C) Validation of promoter replacement by PCR. PCR products obtained with primers BGS1-EXT-FWD and BGS1-INT-REV characteristic of either "WT" HLS1000 (3790 bp) or pZtNIA1::BGS1 CPR transformants (HLS1011 1-3) (5580 bp) were separated by agarose gel electrophoresis. All HLS1011 CPR transformants tested had amplified a 5580 bp band demonstrating that they have integrated pZtNIA1::BGS1 at bgs1 locus. (D) Differential growth of pZtNIA1::BGS1 CPR transformants (HLS1011 1-3) and "WT" HLS1000 (xkU70-44186). After 4 days of growth on MM-Zt+NaNO₃ at 18°C, serial dilutions of yeast-like cells (10⁴, 10⁵ and 10⁶ cells/ml) were spotted on inducing (MM-Zt+NaNO₃) or repressing media (MM-Zt+Glu) and incubated at 18°C for 6 days. With an inoculum of 10⁷ cells/ml, growth of pZtNIA1::BGS1 CPR transformants (HLS1011 1-3) was strongly reduced on MM-Zt+Glu compared to "WT" HLS1000. (E) Hypersensitivity of pZtNIA1::BGS1 CPR transformants to CFW and CR. Serial dilutions of yeast-like cells (10⁴, 10⁵ and 10⁶ cells/ml) were spotted on inducing medium (MM-Zt+NaNO₃) amended with 100 mg/l CFW or CR. Growth of pZtNIA1::BGS1 CPR transformants (HLS1011 1-3) was strongly inhibited in presence of CFW or CR compared to "WT" (HLS1000) at all inoculum dilutions tested.

![Fig. 1. Comparison of pMoNIA1::EGFP and pZtNIA1::EGFP expression in *Z. tritici*. (A) EGFP fluorescence of ZtpELMA-1 and HLS1009-1 transformants grown in inducing conditions. Transformants ZtpELMA-1 (pMoNIA1::EGFP) and HLS1009-1 (pZtNIA1::EGFP) and wild type (IP0323) were grown on MM-Zt+NaNO₃ at 18°C in agitated liquid cultures (180 RPM) for 3 days. Images in bright field (BF) and fluorescence (GFP: 488 nm/50 mW excitation) channels were acquired at 100× magnification and 200 ms exposure using an Olympus spinning disc fluorescence microscope. A strong cytoplasmic fluorescence resulting from EGFP expression was observed in yeast-like cells from transformants ZtpELMA-1 and HLS1009-1. (B) Quantitative analysis of EGFP fluorescence of ZtpELMA-1 and HLS1009-1 transformants grown on different nitrogen sources. Transformants ZtpELMA-1 (pMoNIA1::EGFP) and HLS1009-1 (pZtNIA1::EGFP) and wild type (IP0323) were grown on MM-Zt+NaNO₃ or MM-Zt+Glu at 18°C in agitated liquid cultures (180 RPM) for 3 days. Average cytoplasmic fluorescence signal intensity was calculated from cellular fluorescence intensity after subtraction of background fluorescence. Data shows average cytoplasmic fluorescence signal intensity from 100 individual measurements (50 per biological replicate/strain) per individual measurements (50 per biological replicate/strain). The intensity of EGFP fluorescence was similar in ZtpELMA-1 and HLS1009-1 transformants grown on MM-Zt+NaNO₃ (2600–2800 AU), while this fluorescence was down-regulated by 10-fold on MM-Zt+Glu for both transformants. (C) qRT-PCR analysis of EGFP and Z. tritici NIA1 expression under the control of pMoNIA1 and pZtNIA1. ZtpELMA-1 transformant (pMoNIA1::EGFP, native ZtpNIA1::NIA1) was grown on MM-Zt+NaNO₃, MM-Zt+Glu, MM-Zt+NaNO₃, or MM-Zt+NaNO₃ for 3 days at induced liquid culture at 18°C (180 RPM). qRT PCR was performed using *Z. tritici* TUB1 as reference gene and the 2^-ΔΔCt method for calculating relative expression levels. Data represent means and standard deviations from three biological replicates.
Similarly, heterokaryotic transformants of *C. posadasii* carrying a *bgs1* deletion could not be purified into haploid strains (Kellner et al., 2005). These results suggest that *BGS1* is essential for growth. However, recent data reported for *A. fumigatus* contradict this hypothesis, since viable haploid *bgs1* deletion mutants were obtained (Dichtl et al., 2014). Still, *A. fumigatus bgs1* deletion mutants displayed abnormal hyphal morphology, reduced mycelial growth and hypersensitivity to CFW and CR. A compensatory mechanism was observed in these mutants leading to an increase in chitin and galactosamino-galactans cell wall contents. *BGS1* silencing experiments were also performed in other fungi and transformants with a lower level of expression of *BGS1* displayed either a reduced growth (*C. graminicola*: Oliveira-Garcia and Deising, 2013) or a hypersensitivity to CFW (*Metarhizium acridum*: Yang et al., 2011). *C. graminicola* and *Z. tritici* displayed the strongest reduction in growth when *BGS1* was down-regulated suggesting that they may lack compensatory mechanisms such as those observed in *A. fumigatus bgs1* deletion mutants.

We have also observed that *p2Nia1::BGS1* CPR transformants displayed a strong hypersensitivity to CFW in inducing conditions. This hypersensitivity could result from lower *BGS1* expression in CPR transformants than WT, leading to cell wall modifications not detrimental for growth but essential for CFW resistance. Whatever the explanation, this observation suggests that the use of conditional promoters with different levels of transcription in *BGS1* displayed a strong hypersensitivity to CFW in inducing conditions. *A. Sharon, Humana Press* (5), 179–185.

**References**


Appendix 7

Construction and high-throughput phenotypic screening of Zymoseptoria tritici over-expression strains

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A R T I C L E   I N F O

Article history:
Received 16 January 2015
Revised 20 March 2015
Accepted 15 April 2015

Keywords:
Zymoseptoria tritici
Functional genomics
Over-expression
High-throughput screening

A B S T R A C T

Targeted gene deletion has been instrumental in elucidating many aspects of Zymoseptoria tritici pathogenicity. Gene over-expression is a complementary approach that is amenable to rapid strain construction and high-throughput screening, which has not been exploited to analyze Z. tritici, largely due to a lack of available techniques. Here we exploit the Gateway cloning technology for rapid construction of over-expression vectors and improved homologous integration efficiency of a Z. tritici Akb7/0 strain to build a pilot over-expression library encompassing 32 genes encoding putative DNA binding proteins, GTPases or kinases. We developed a protocol using a Rotor-HDA robot for rapid and reproducible cell pinning for high-throughput in vitro screening. This screen identified an over-expression strain that demonstrated a marked reduction in hyphal production relative to the isogenic progenitor. This study provides a protocol for rapid generation of Z. tritici over-expression libraries and a technique for functional genomic screening in this important pathogen.

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1. Introduction

Gene disruption or deletion has been an essential technique for understanding the molecular basis of Zymoseptoria tritici virulence, including the role of MAP kinase signaling (Cousin et al., 2006; Mehrabi et al., 2006), transcription factor regulation (Kramer et al., 2009; Mirzadi Gohari et al., 2014), transport (Stergiopoulos et al., 2003) and effector biosynthesis (Marshall et al., 2011). In the rice blast fungus Magnaporthe oryzae, deletion of every gene encoding the autophagic apparatus clearly demonstrates that gene inactivation is a strategy amenable to functional genomics of plant pathogens (Kershaw and Talbot, 2009), and large-scale gene knock-out libraries have proven invaluable for elucidation of function in model and pathogenic fungi (Goncalves et al., 2011; Schwarzmuller et al., 2014). However gene deletion approaches have certain limitations. For example, essential genes cannot be functionally characterized by deletion. Additionally, for processes where multiple genes of similar function act synergistically, functional redundancy may make time-consuming characterization of null isolates ineffective. Moreover, phenotypic variation of nulls relative to wild-type isolates may be difficult to identify if the gene is not transcribed in standard laboratory culture, or transiently expressed at cryptic stages during infection assays.

Gene over-expression is a complementary approach to gene disruption or deletion. For infectious diseases of plants, researchers have utilized powerful heterologous expression technology facilitated by the availability of genetically tractable vectors and/or host systems. For example, a library of Cladosporium fulvum cDNAs was expressed in Agrobacterium tumefaciens, and four hypersensitive response-inducing genes were identified following leaf inoculation (Takken et al., 2000). Similarly, Agrobacterium mediated over-expression of Phytophthora infestans effectors in planta has revealed numerous responses by the host, including disease resistant hypersensitivity (Oh et al., 2009; Vleeshouwers et al., 2008). In addition to studies that probe the host/pathogen interface, heterologous expression has been implemented in model organisms, which is exemplified by work defining the Z. tritici CYP51 gene in Saccharomyces cerevisiae (Cools et al., 2010).

In addition, over-expression of genes within the native organism has been used to elucidate basic biology for both model microorganisms and pathogens, especially when combined with high-throughput library construction and screening. Jin and colleagues used 2043 over-expression isolates in conjunction with 3627 transposon insertion mutants to identify the role of mitochondrial function in pseudohyphal growth of S. cerevisiae (Jin et al., 2008). In the pathogen Candida albicans, screening of a 257
open reading frame over-expression library identified a novel role in morphogenesis for 11 genes (Chauvel et al., 2012).

The aim of this study was to provide proof of principle for generating a genome-wide Z. tritici over-expression library and to develop a high-throughput technique for rapid functional screening in vitro. Accordingly, genes enriched amongst putative DNA binding proteins, kinases or GTPases were PCR amplified and cloned into pDONR207 to generate Gateway™Entry plasmids. Genes were subsequently shuttled into the newly described Gateway®Destination vector pYSKH3 (Sidhu et al., 2015). These A. tumefaciens adapted vectors were used to place genes under control of the Z. tritici translation elongation factor (tef), MycG3C92705 promoter at the disrupted Aku70 locus in strain HLS1000. The pilot library containing 32 Z. tritici over-expression strains was used to optimize a high-throughput screening protocol, where in vitro grown cells were rapidly and reproducibly pinned, using a Singer Rotor-HDA robot, onto solid agar containing various abiotic stressors that mimic stresses that might be encountered in the host. Using this approach, we identified an isolate that produced markedly less hyphae relative to the isogenic progenitor strain at the colony periphery under several stress conditions. This study provides a robust protocol for rapid generation of Z. tritici over-expression libraries and for high-throughput functional genomic screening. We also demonstrate proof of principle that this genome wide functional analysis will enable discovery of novel infection related biology.

2. Methods

2.1. Growth media

Z. tritici synthetic complete (ZTSC), a defined rich growth medium, comprising 6.9 g/l yeast nitrogen base without amino acids (ForMedium, UK), 0.79 g/l complete supplement mix (ForMedium), 20 g/l glucose, 20 g/l bacteriological agar (Lab, UK). Czapex Dox, a defined nutrient limiting medium, comprising 33.4 g/l Czapex Dox (Oxoid, UK), 20 g/l bacteriological agar (Lab), Bacteria were grown in LB medium (Formedium) supplemented with kanamycin salt at 50 µg/ml (Sigma, UK) or gentamicin at 50 µg/ml (Sigma) where appropriate.

All strains were routinely stored at -80°C in 50% (v/v) glycerol.

2.2. Plasmids used in this study

All plasmids were stored at -20°C prior to use. Gateway™Entry vectors were constructed using pDONR207 (Invitrogen, UK) which contains a gentamicin resistance gene for selection in Escherichia coli. This plasmid also contains a ccdB gene flanked by attR sequences for Gateway® mediated recombination. For construction of over-expression vectors, we used pYSKH3 (Sidhu et al., 2015). This vector confers kanamycin resistance for selection in E. coli and A. tumefaciens. pYSKH3 contains a ccdB gene (co-ordinates 14,856–16,558) flanked with attR sites for Gateway® mediated recombination and selection. With regards to gene over-expression in Z. tritici, the Gateway® ccdB gene is flanked by an upstream sequence encoding (S’–S’): 26 bp t-border repeat (8733–8758), 1000 bp homology region to 5’UTR of the Z. tritici Ku70 locus (8854–9853), 2361 bp functional Ku70 gene (9854–12,214), 1382 bp of hygromycin resistance cassette (12,243–13,625) and 1200 bp translation elongation factor promoter (13,656–14,855). The 3’ region of the ccdB gene is flanked by a stop codon, 212 bp CYC1 terminator (16,634–16,873), 800 bp region of 3’ UTR of Ku70 locus (16,980–17,779) and a 26 bp t-border repeat (18,094–18,119, see Fig. 1A). Derivatives of pYSKH3 in which the ccdB gene was replaced with a DNA sequence encoding a Z. tritici gene were named pCCKH and numbered 1-32 (Supplementary Table S1).

2.3. Strains used in this study

Z. tritici strain HLS1000 (Sidhu et al., 2015) was used throughout. In this IPO323 derivative, the Ku70 gene (MycG3C85040) of Z. tritici has been replaced with a G418 cassette using A. tumefaciens mediated transformation.

E. coli One Shot® ccdB Survival™2 T1® was used for propagation of pDONR207 (Invitrogen, UK) and pYSKH3 (Sidhu et al., 2015). All Gateway®Entry and modified destination vectors were propagated in DH5a (Invitrogen, UK).

The kanamycin sensitive A. tumefaciens strain EHA105 (Hood et al., 1993) was used for Z. tritici transformation.

2.4. Construction of Gateway Entry vectors

For PCR amplification of each gene of interest, forward primers were designed to include the attB1 site (gggagactttgtaaaaaacagcgttggct) and the first 20 bp of the gene, and reverse primers to include the attR2 site (gggagactttgtaaaaaacagctggttc). Primers were synthesized by Sigma (Table S1). PCR was conducted using Phusion® High-Fidelity DNA Polymerase (NEB) with a 65°C primer annealing temperature and an extension of 0.5 min/kb, using Z. tritici IPO323 genomic DNA as template. PCR amplicons of predicted sizes were confirmed by gel electrophoresis, PEG purified, suspended in 10 µl TE buffer (40 mM TRIS base, 20 mM glacial acetic acid, 0.1 mM EDTA, pH 8). For construction of Gateway®Entry vectors, 150 ng of pDONR207 was mixed with 2.5 µl of purified PCR product. 1 µl of Gateway® BP Clonase™ with TE buffer added to a total volume of 10 µl. Reactions were incubated at 25°C for 24 h then treated with Proteinase K (Invitrogen, UK) following the manufacturer’s instructions. E. coli strain DH5α was transformed with 5 µl of each reaction mixture. Transformants were selected on LB supplemented with gentamicin (50 µg/ml). Colonies were grown over-night in LB medium with selection, and plasmids extracted using Plasmid Mini Kit (Qiagen, UK). In order to confirm recombination of the ccdB gene with the gene of interest, plasmids were digested with BsrGI (NEB, UK), and digest reactions analyzed by gel electrophoresis. Putative Gateway®Entry vectors were Sanger Sequenced (Eurofins, UK) using primer GOXF (tcggcttaacgctagcatgga).

2.5. Generation of Z. tritici over-expression plasmids

For construction of over-expression vectors pCCKH 1–32, 200 ng of Z. tritici Gateway®Entry plasmids were mixed with 200 ng of pYSKH3, 1 µl of LR Clonase™ with TE buffer added to a total volume of 10 µl. Reactions were incubated at 25°C for 24 h then treated with Proteinase K (Invitrogen) following the manufacturer’s instructions. Reaction mixtures were transformed into E. coli strain DH5α as described above except transformants were selected on LB medium supplemented with kanamycin (50 µg/ml). Colonies were grown over-night in LB medium with selection, and plasmids extracted using Plasmid Mini Kit (Qiagen, UK). In order to confirm recombinant of the ccdB gene with the gene of interest, plasmids were digested with BsrGI (NEB, UK), and digest reactions analyzed by gel electrophoresis. Putative Gateway®Entry vectors were Sanger Sequenced (Eurofins, UK) using primer GOXF (tcggcttaacgctagcatgga).

2.6. PCR confirmation of Z. tritici over-expression isolates

Genomic DNA was isolated from each putative transformant and integration of the over-expression construct at the Aku70::G418 locus determined by two PCR reactions (Figs. 2A...
Firstly, forward primer Term_F (gtcgaagctgcttataatgc) and reverse primer Ku70_EXT_R (ctggacatcaagcttcggat) yield a 1.8 kb product which is restricted to gDNA templates where the $Aku70$:G418 locus has been replaced with a pCCKH over-expression cassette. Genomic DNA templates were further screened with forward primer Hyg_F (cttcttgagggctcggtt) and Ku70_EXT_R. These primers span a 1.57 kb 5' region of the cassette, followed by sequence encoding the protein of interest and finally a 2.11 kb 3' region. Accordingly, the size of the PCR product varies for each over-expression strain (Fig. 1A). This second PCR was used as a final screen to verify the gene of interest was integrated at the $ku70$ locus. Single integration events were confirmed for eight transformants by Southern blot analysis (see Supplementary Methods S2).

2.7. High throughput phenotypic screen

2.7.1. Preparation of cells and target/source plates

Robotic screening consisted of multiple rounds of pinning from a 96 well liquid cell suspension (source plate) onto multiple solid agar (target) plates. For the source plate, the Z. tritici HLS1000 $Aku70$ progenitor strain and pCCKH over-expression isolates were obtained from long term storage at -80 °C in 50% (v/v) glycerol and grown for 7 days at 18 °C on ZTSC medium. Budding cells were scraped from plates using a sterile loop and added to 500 μl water, and washed by centrifugation at 5000 RPM for 10 min. Cells were re-suspended in water and cell density adjusted to 5 x 10^6 cells/ml. Then 150 μl aliquots were arrayed in duplicate wells of a 96 well plate. Target agar plates consisted of 42 mls of ZTSC medium in Plus Plates (Singer Instruments, UK). For stress conditions, media was cooled to 42 °C and supplemented with hydrogen peroxide (1 mM, 2 mM, 5 mM), calcofluor white (25 μg/ml, 50 μg/ml), congo red (200 μg/ml/400 μg/ml) or caspofugin acetate (2.5 μg/ml). Technical duplicates were conducted for each condition with triplicate biological repeats.

2.7.2. Robotic pinning

Robotic pinning was conducted with a Rotor-HDA pinning robot (Singer Instruments) using manufacturer's software. The source plate was arrayed onto target agar plates using Repad 96LW pins (Singer Instruments). For collection of cells from source plate, a speed of 19 mm/s and plate retraction of 0.5 mm was used. Mixing of cell suspension between pinning was 3 rotations of 1.3 mm diameter at a speed of 25 mm/s. To ensure appropriate contact with the agar surface, the following parameters were used: pin pressure 22%, speed 15 mm/s, overshoot 1.9 mm, clearance 0.5 mm, diameter 1 mm. Using these parameters, a total of ~1000 Z. tritici cells in 2 μl liquid suspension are pinned per sample.

2.7.3. Imaging colony growth

Colonial growth was observed daily. Images were captured using a M205FA (Leica, UK) stereo microscope at various magnifications using Leica application suite v 3.8.0. A V750 Pro Scanner (Epson, UK) was used for imaging gross plate layout.

2.8. Manual phenotypic screening

For manual reproduction of phenotypes detected by robotic pinning, cells were prepared as described above and serially diluted 1:10 in sterile water from densities 1 x 10^5–1 x 10^6 cells/ml. A total of 10 μl of each cell density was added to 25 ml agar plates containing concentrations of abiotic stressors indicated above. For quantification of strain growth rates on defined nutrient limiting medium, cell density was adjusted to 200 cells/ml and 50 μl plated onto 25 ml Czapex Dox agar plates. Samples were incubated for 14 days at 18 °C, after which 50 colonies/strain were imaged using a Leica M205FA stereo microscope. Scale bars were added using Leica application suite v 3.8.0, images imported to ImageJ version 1.48v (NIH, Maryland, USA) and pixel/μM ratio calibrated. Colony area was measured by manually drawing around colony perimeter and recorded as mm²/colony. Triplicate biological replicates were conducted.

3. Results

3.1. Development of a pilot over-expression library in Z. tritici

In order to construct over-expression vectors for A. tumefaciens mediated transformation of Z. tritici, we utilized the Gateway® experimental pipeline (see Sections 2.4 and 2.5). Forty genes were selected based on GO terms from JGI protein annotation (Supplementary Table S2). DNA sequences were PCR amplified with attR1/attR2 sites at the 5’ and 3’ flanks respectively, and recombined into donor plasmid pDONR207. All putative genes were successfully cloned using this approach, then subsequently recombined into Gateway® adapted destination vector pYSKH3 for A. tumefaciens mediated transformation of Z. tritici. In the pYSKH3 vector, genes are under 5’ control of the gfp1 promoter.
and followed immediately by a 3’ stop codon and terminator (Fig. 1A). Additionally, this vector has the following functionality in Z. tritici: (a) the over-expression cassette is targeted to the \(\Delta ku70\)-G418 locus; (b) the Ku70 gene (Mycgr3g111569) is reconstituted, and (c) the selection marker for transformation is hygromycin (Fig. 1B). Following Z. tritici transformation, hygromycin resistant isolates were recovered and PCR used to confirm integration of the over-expression cassette at the target locus (Fig. 1C). A total of 32 Z. tritici strains with tef1 promoter directed over-expression of different genes at the ku70 locus were generated using this methodology. Southern blot analyses (\(n=8\)) confirmed single integration of the pCCKH cassette in recipient genomes (see Supplementary Fig. S4).

3.2. High throughput phenotypic screening of a Z. tritici over-expression library on oxidative and cell wall stress conditions

Given that Z. tritici infection is initiated on the leaf surface, we developed a high throughput assay for monitoring growth on solid substrates. This approach optimized a technique where isolates are robotically pinned onto agar plates +/- stress agents and growth compared. Stress conditions for library screening were selected in order to perturb Z. tritici processes which we hypothesize are required for infection. The fungal cell wall is a critical mediator of host-pathogen interactions, and extensive remodeling is required during germination and other differentiation events during pathogenesis. In order to determine genes for which tef1 based expression at the ku70 locus resulted in modulation of cell wall biosynthesis or architecture, isolates were screened on ZTSC agar plates supplemented with congo red or calcofluor white. These dyes perturb cell wall biosynthesis and integrity by binding chitin (Ram and Klis, 2006). Additionally, medium supplemented with caspofungin acetate was utilized to screen for genes which impact 1,3-beta-glucan synthase activity and/or antifungal resistance. Finally, we used hydrogen peroxide to probe for genes that respond to host derived oxidative stress (Shetty et al., 2007).

Strains were arrayed in technical duplicate onto solid agar using a Rotor-HDA pinning robot and colony morphology monitored for 16 days (Figs. 2A and 2B). The 32 over-expression isolates and \(\Delta ku70\) control could be pinned onto target plates at approximately 4 plates/minute. Approximately 1000 cells per technical sample were pinned onto ZTSC agar and grown at 18°C (see Section 2.7). A defined growth medium was used in order to avoid ambiguity with regards to nutritional content and to reduce variation between batches of media. Melanisation, which might impact sensitivity to numerous abiotic stressors, was not observed (Fig. 2B). Colony morphology on ZTSC plates reproducibly consisted of an area of mucoid budding cells surrounded by hyphae at the colony periphery (Figs. 2A and 2B). We therefore reasoned that this assay can be used to probe both budding and mycelial Z. tritici morphologies.

The progenitor and over-expression isolates demonstrated indistinguishable growth rates and colony morphology when grown on ZTSC medium (Figs. 2 and 3), indicating that homologous replacement of the ku70 locus with pCCKH vectors does not detectably impact in vitro growth on defined rich medium. Furthermore, 31 over-expression isolates displayed comparable growth/morphology to the HLS1000 progenitor control on the various stress conditions tested, indicating tef1 directed expression of these genes does not impact upon susceptibility to these compounds in this assay. However, the isolate HLS1108 displayed a striking reduction of hyphal growth at the colony periphery relative to progenitor control and other over-expression strains when perturbed with \(H_2O_2\), congo red and calcofluor white (Figs. 2 and 3). The gene over-expressed in this isolate encodes a putative transcription factor (Mycgr3g111569, UniProt accession: F9XPA7). Interestingly, mucoid colony growth was not affected by stress conditions for this isolate. Indeed, under caspofungin acetate stress which completely inhibited hyphal growth, control and HLS1108 strains demonstrated comparable colony morphology (Fig. 3). We therefore hypothesize that (a) tef1 directed expression of gene Mycgr3g111569 results in Z. tritici hyphal sensitivity to both oxidative and chitin mediated cell wall stressors; however, (b) budding growth is not detectably impacted by Mycgr3g111569 over-expression.

3.3. Manual confirmation of phenotypes identified by robotic screen

In order to confirm that phenotypes observed during high-throughput robotic screening were representative of standard in vitro experimentation, hyphal sensitivity of strain HLS1108 was investigated in simple manual assays. Budding cells were serially diluted to densities of \(1 \times 10^5 \text{–} 1 \times 10^7\)/ml and pipetted onto agar containing ZTSC +/- stress conditions. Strains HLS1000 and HLS1127 were used as controls for isogenic progenitor strain and pCCKH modified isolates respectively. Following growth at 18°C for 18 days, morphologies of all three strains were indistinguishable on ZTSC medium, but HLS1108 demonstrated reduced hyphal production on ZTSC medium + \(H_2O_2\), congo red or calcofluor white relative to controls (Fig. 3b). We therefore consider the high
throughput technique described above to be sufficiently similar to low throughput experimentation to allow robust hypothesis generation.

Given that over-expression strain HLS1108 demonstrated a hyphal sensitivity phenotype to cell wall and oxidative stressors, we reasoned that this gene may be antagonistic to Z. tritici hyphal growth. In order to test this hypothesis, Δku70, HLS1127 controls and HLS1108 budding cells were inoculated onto Czapex Dox medium at a density of 1000 cells on either ZTSC medium or ZTSC supplemented with 2 mM H2O2. Colony morphology between control and stress conditions were identical at this time point for all strains except HLS1108, which demonstrated marked reduction in hyphal production at the colony periphery. Image was captured using an Epson V750 Pro Scanner.

strain HLS1108 relative to control strains, which was highly statistically significant (Student’s T-test p < 0.0001, Fig. 4). No significant difference was observed between the progenitor and pCCKH modified controls. Microscopic examination revealed a reduction of hyphal production around the colony periphery in the over-expression isolate (Fig. 4). These data suggest that tef1 driven over-expression of putative transcription factor Mycgr3g111569 impedes hyphal production of Z. tritici. We name this gene almA, for a lot less mycelium A, and consider the over-expressing strain a useful tool for reverse engineering the molecular basis of hyphal growth in Z. tritici.
4. Discussion

The first transformation of *Z. tritici* was reported in 1998 (Payne et al., 1998), and a recent review by Orton and colleagues summarized the 23 genes that had been functionally characterized by 2011 (Orton et al., 2011). Despite revealing significant insights into *Z. tritici* biology, at this current rate of progress it will be approximately 50 years before 1% of the products of the 10,933 predicted *Z. tritici* genes have been functionally characterized. In order to improve the rate of functional characterization of *Z. tritici* genes, we provide proof of principle for generating a *Z. tritici* over-expression library and describe a high throughput technique for rapid phenotypic screening in *vitro*.

Construction of a *Z. tritici* over-expression library utilized the Gateway cloning experimental pipeline for rapid vector construction, and a ku70 null isolate, HLS1000, for improved homologous recombination during *Z. tritici* transformation. Using these tools we were able to successfully integrate 80% of genes at the ku70 locus under control of the tef1 promoter, which is a pass rate consistent with similar studies in other pathogenic fungi (Chauvel et al., 2012). The molecular tools used for library construction confer additional functionality to mitigate both false positive and false negative results that might confound interpretation of data from high throughput functional genomic screens. Firstly, targeting over-expression cassettes to the ku70 locus is preferable to random integration, which might lead to disruption of other coding sequences, or occur at transcriptionally silent/and or dispensable regions of the *Z. tritici* genome. Additionally, while the *Z. tritici* ku70 null mutant used in this study improves homologous targeting, genome instability in this strain may introduce phenotypic aberrations in derivative isolates. Genome instability is a well documented phenotype of ku strains, which is supported by sensitivity of the Δku70 isolate to ultraviolet directed DNA damage (Sidhu et al., 2015). Accordingly, vector pYSKH3 contains the full coding sequence of the wild-type *Z. tritici* Ku70 gene (Mycgr3G85040) to restore wild-type levels of genome stability.

Next, a high throughput in *vitro* screening protocol was optimized for a Rotor-HDA pinning Robot (Singer Instruments). Using this platform, 96 well cell suspensions can be arrayed on to four agar plates per minute. This assay is simple to scale up by using multiple 96 well source plates and/or increasing the number of conditions probed by target plates. Although beyond the scope of this study, we are optimizing in *vitro* conditions which probe other possible elements of *Z. tritici* niche adaptation during infection, such as macro and micro nutrient depletion, desiccation and osmotic fluctuations. While we have screened over-expression isolates, it would be easy to screen other *Z. tritici* libraries, such as naturally occurring wild-type strain collections or large numbers of gene knock-outs. Additionally, it will be simple to adopt the platform to liquid growth assays by changing target agar plates to 96 well liquid suspensions.

While functional characterization of genes by over-expression confers several advantages to gene disruption or deletion, such as facilitating characterization of essential genes and avoiding issues
of functional redundancy, there are certain limitations. For example, feedback mechanisms required for proper transcription factor function during native expression might be distorted by significantly elevated protein levels. For kinases, over-expression can lead to inactive protein subcomplexes (Gibson et al., 2013). More specifically for this study, while tef1 controlled over-expression of pYSKH3 derivatives in Z. tritici has recently been validated using a GFP reporter (Sidhu et al., 2015), as protein levels were not confirmed in this study we cannot rule out that post transcriptional silencing occurred for certain genes. Additionally, we assume that the tef1 promoter is constitutively active, although the activity of this promoter might vary for certain stress conditions. Therefore, differences between the tef1 promoter activities between experimental conditions might explain why strain HLS1108 maintained hyphal production at the colony periphery when grown on synthetic complete medium but not on Czapex Dox or stress conditions. Notwithstanding these limitations, the high throughput screen described in this study demonstrated over-expression of the putative Z. tritici transcription factor almA results in hyphal sensitivity to oxidative and chitin based cell wall stress. A simple growth assay on hyphal inducing conditions demonstrated a reduction in hyphal growth in this strain relative to the progenitor strain. We are currently working to identify the downstream targets for this transcription factor, which might identify important attributes of Z. tritici hyphal growth or architecture.

5. Conclusion

This study has presented the construction of a library of Z. tritici over-expression strains and developed a high throughput in vitro screen. The identification of a mutant with reduced hyphal production provides proof of principle that these techniques will facilitate genome-wide functional genomics analysis of Z. tritici pathogenicity via over-expression screens, and reveal novel biological functions.

Acknowledgments

This work was funded by a BBSRC BBR grant (BB/I025956/1) to KH and collaborators and a BBSRC CASE studentship (BB/J500793/1), supported by Syngenta UK.
Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2015.04.013.

References


