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

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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 sulfonyleurea 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 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 $\leq 10\%$ seen in the wild type 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 (Polley 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 pycnidial 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 effector proteins (Motteram et al., 2009), mitogen activated protein kinases (Mehrabani 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

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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; Zwiars 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 *MgSDHB* 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. Additionally the geneticin resistance marker was used in a previous study to inactivate the *Z. tritici* *KU70* 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; Sweigard 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 (*ILV2^{SUR}* (GenBank accession AF013601), based on an allele of the *Magnaporthe oryzae* *ILV2* gene, which encodes a variant of the acetolactate 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.cbs.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 ammonium 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 Naqvi, 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 μ M FeSO₄, 4 mM (NH₄)₂SO₄, 10 mM Glucose, 40 mM 2-N-morpholino ethanesulfonic acid, 0.5% Glycerol (w/v), 15 g agar/litre (w/v); pH to 5.6 with 1 M NaOH) supplemented with 200 μ M acetosyringone was used for ATMT. For the mutagen sensitivity screen, *Z. tritici* strains were inoculated in synthetic complete (SC) broth (SC broth per litre: 6.9 g yeast nitrogen base 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 DH5 α was used to propagate all plasmids. *E. coli* cultures were grown at 37 °C in Luria–Bertani (LB) media supplemented with 100 μ g/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 μ g/mL kanamycin and 100 μ g/mL rifampicin.

2.2. Nucleic acid manipulations

Molecular cloning and DNA manipulations were carried out by 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 disruptor (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 pC-HYG (Motteram et al., 2009) was modified by addition of the *S. cerevisiae* 2 μ origin of replication and the *URA3* selection marker to construct ternary vector pC-HYG-YR (Addgene ID – 61765) (see supplementary materials and methods). The ternary vectors pC-SUR-YR (Addgene ID – 61768), pC-BAR-YR (Addgene ID – 61766) and pC-G418-YR (Addgene ID – 61767) in which the hygromycin resistance marker of pC-HYG-YR was replaced with the markers which confer resistance against sulfonylurea, glufosinate ammonium and geneticin, 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 pC-HYG-36951-KO, pC-HYG-72646-KO and pC-HYG-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 pC-HYG-YR and yeast recombinational cloning (see supplementary materials and methods).

2.4. *A. tumefaciens* mediated transformation of *Z. tritici*

The vectors pC-G418-KU70-KO, pC-G418-KU80-KO, pC-SUR-KU80-KO, pC-HYG-36951-KO, pC-HYG-72646-KO, pC-HYG-102083-KO and pC-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 (Zwiers 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 \times 10⁷ cells/mL in sterile water) were mixed in a ratio of 3:1 and 400 μ L 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 μ g/mL sulfonylurea or YPD agar containing 200 μ g/mL geneticin (G418 sodium salt) or hygromycin B and antibiotics (100 μ g/mL ampicillin, 100 μ g/mL cefotaxime, 100 μ g/mL streptomycin and 100 μ g/mL timentin). After 14 days putative drug resistant *Z. tritici* transformants were sub cultured for two rounds of selection on BM agar containing 10 μ g/mL sulfonylurea or YPD agar containing 200 μ g/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:Sur^R 1–15. The genomic integration of the

sulfonylurea resistance cassette in strains IPO323:Sur^R 1–15 was confirmed by diagnostic PCR using primers pSUR^R-FWD and SUR^R-INT-R which amplify a single 558 bp fragment specific to the resistance cassette.

Putative *Z. tritici* *ku70* and *ku80* null strains resistant to geneticin and harbouring pC-G418-KU70-KO and pC-G418-KU80-KO were isolated. These strains were designated HLS1000 ($\Delta ku70:G418^R$) and HLS1001 ($\Delta ku80:G418^R$) 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 ($\Delta ku80:Sur^R$). 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^R-INT-R, KU80-EXT-F/KU80-INT-R/G418^R-INT-R or KU80-EXT-F/KU80-INT-R/SUR^R-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 *HindIII* 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 geneticin 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/HYG^R-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/HYG^R-INT-R and 102083-EXT-F/HYG^R-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:Sur^R 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 μ L aliquot of cell

suspension (serially diluted to 10^6 , 10^5 and 10^4 cells/mL in sterile water) was spotted in triplicate on BM agar containing sulfonyleurea concentrations ranging from 0 to 12 $\mu\text{g}/\text{mL}$. 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 (1×10^7 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

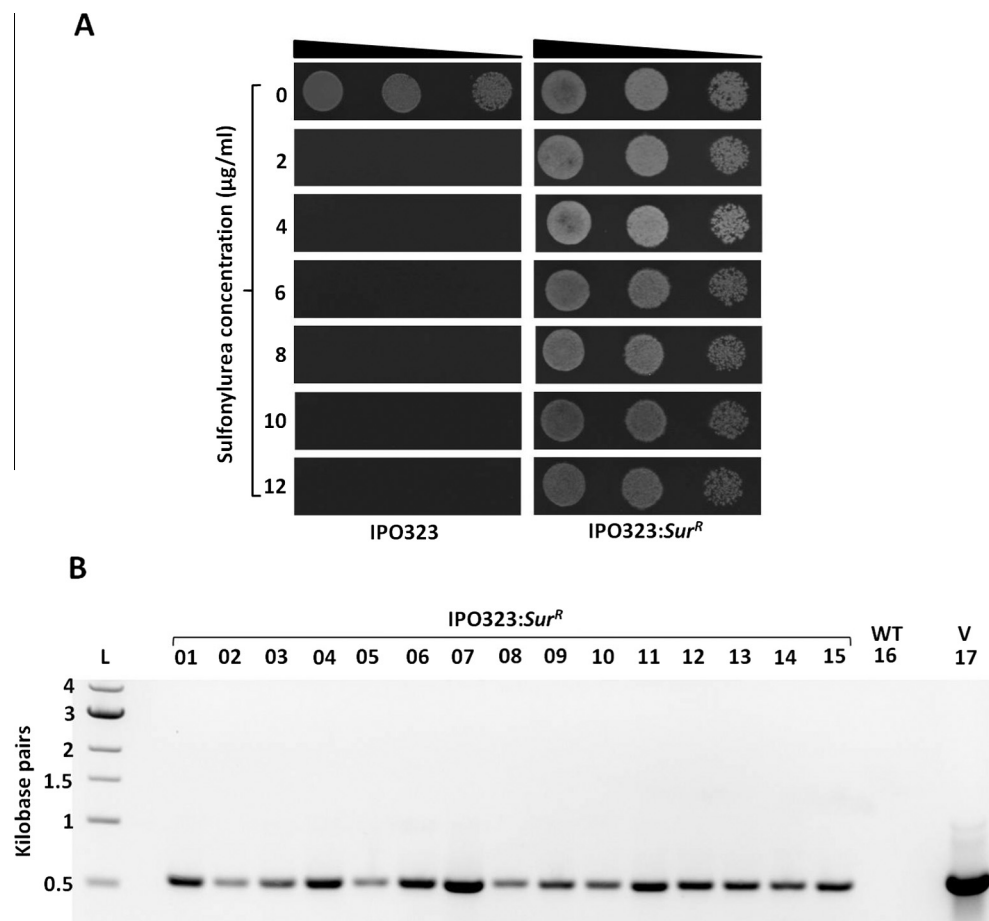


Fig. 1. *Z. tritici* wild type strain IPO323 is hypersensitive to sulfonyleurea (A) and genomically integrated *ILV2^{SUR}* sulfonyleurea resistance cassette confers resistance to the drug (B). (A) *Z. tritici* wild type strain IPO323 is hypersensitive to sulfonyleurea (chlorimuron ethyl is the active ingredient) at low concentrations (2 $\mu\text{g}/\text{mL}$) while strains IPO323:*Sur^R* (independent mutants containing a randomly integrated sulfonyleurea resistance cassette) are drug resistant following 10 rounds of growth on non-selective media. *Z. tritici* cells (from 10^6 to 10^4 cells/ml) were spotted on BM agar supplemented with various sulfonyleurea concentrations and plates were incubated at 18 °C for 6 days. The wild type strain IPO323 shows severe growth inhibition whereas the IPO323:*Sur^R* mutant is resistant to sulfonyleurea concentrations up to 12 $\mu\text{g}/\text{mL}$. (B) Agarose gel (1% w/v in TAE) image showing PCR confirmation of presence of the sulfonyleurea resistance cassette in the genomic DNA isolated from 15 randomly selected IPO323:*Sur^R* mutants (Lane 1–15). Amplification of a 558 bp PCR product confirms that the sulfonyleurea resistance cassette is present in strains IPO323:*Sur^R* 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 sulfonyleurea 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^{SUR}* sulfonyleurea resistance cassette from *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 sulfonyleurea and functionality of the resistance cassette in *Z. tritici*

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

To confirm the functionality of the sulfonyleurea resistance cassette in *Z. tritici*, the vector pC-SUR-YR was transformed into strain IPO323 and 15 sulfonyleurea resistant transformants designated strain IPO323:Sur^R 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 sulfonyleurea resistance cassette was amplified from strains IPO323:Sur^R 1–15 but the PCR product was absent in case of wild type *Z. tritici* IPO323 (Fig. 1). Further, a single IPO323:Sur^R strain was sub-cultured for 10 rounds on non-selective medium and then spotted on BM agar containing 1–12 µg/ml sulfonyleurea (Fig. 1). This strain retained drug resistance suggesting genomic integration of the sulfonyleurea resistance cassette was mitotically stable even in absence of selection

pressure. These data demonstrate that the sulfonyleurea resistance cassette from *M. oryzae* is a functional dominant selection marker in *Z. tritici*.

3.3. Inactivation of the *Z. tritici* *KU70* and *KU80* genes

To inactivate the predicted *Z. tritici* *KU70* (Gene ID Mycgr3G85040) and *KU80* (Gene ID Mycgr3G40048) 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 *KU70* by the geneticin resistance cassette (Fig. 2A), using the primer pairs *KU70*-EXT-F/*KU70*-INT-R and *KU70*-EXT-F/*G418^R*-INT-REV. Out of 96 putative transformants tested, the amplification of a 2635 bp product in three suggested the replacement of *KU70* 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 *ku70* null strains also suggested the presence of a wild type *KU70* allele (Fig. 2C). 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 (Fig. 2B). A 3228 bp PCR product amplified using primers *KU80*-EXT-F/*G418^R*-INT-REV from the genomic DNA isolated from three mutants suggested the *KU80* 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 *ku80* null mutants suggested the presence of wild type *KU80* gene (Fig. 2D). Southern blot analysis confirmed that in the *ku70* and *ku80* null strains the target genes had been replaced by the geneticin resistance cassette without any ectopic integrations events (Fig. S2). These confirmed *ku70* and *ku80* null strains were designated *Z. tritici* HLS1000 and HLS1001, respectively.

We also attempted to inactivate *Z. tritici* *KU80* with pC-SUR-*KU80*-KO to test the utility of the sulfonyleurea resistance

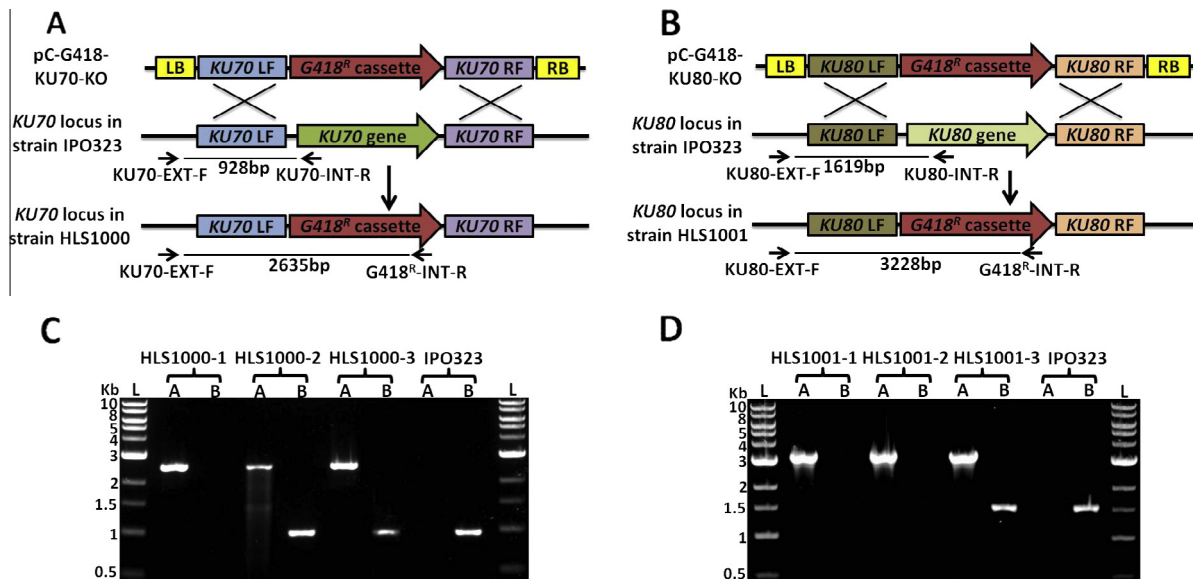


Fig. 2. Construction of *Z. tritici* *ku70* and *ku80* null strains. *Z. tritici* *KU70* and *KU80* gene deletion vectors pC-G418-*KU70*-KO and pC-G418-*KU80*-KO were introduced into the wild type strain IPO323 by *A. tumefaciens* mediated transformation. Schematic representations of the gene deletion strategies for *Z. tritici* *KU70* (A) and *KU80* (B) are shown. *A. tumefaciens* left (LB) and right (RB) border regions (yellow) on the transfer DNA (TDNA) flank the deletion constructs. Homologous recombination between sequences in the left flank (*KU70*LF or *KU80*LF) and right flank (*KU70*RF or *KU80*RF) of the deletion cassette and the chromosomal *KU* loci mediate integration into their respective target locus to replace *KU70* and *KU80* with the geneticin resistance marker (*G418^R* cassette) resulting in *ku70* and *ku80* null strains HLS1000 and HLS1001, respectively. (C and D) Image of ethidium bromide stained agarose gel (2% in TAE (w/v)) shows the results of the diagnostic PCR on putative *ku70* and *ku80* null strains. Primer pair *KU70*-EXT-F/*KU70*-INT-R and *KU70*-EXT-F/*G418^R*-INT-R amplify 928 bp and 2635 bp products which confirm the presence of *KU70* wild type gene and replacement of the *KU70* by the geneticin resistance marker, respectively. Similarly, primer pairs *KU80*-EXT-F/*KU80*-INT-R and *KU80*-EXT-F/*G418^R*-INT-R amplify a 1619 bp product specific to the *KU80* wild type gene and amplification of a 3228 bp product indicates replacement of the *KU80* gene by the geneticin resistance marker. The NEB 1 kb DNA ladder (L) was used as a standard. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Gene targeting frequency in *Z. tritici* strains HLS1000 (*ku70* null), HLS1001 (*ku80* null) and IPO323 (wild type). (Frequency was measured as percentage of integrations into target locus).

Gene name/ID	Size (bp)	Locus	HLS1000 (%)	HLS1001 (%)	IPO323 (%)
Mycgr3G36951	13,961	chr_2:439283–453243	93.75	100	6.25
Mycgr3G72646	7,932	chr_5:2631468–2639399	100	87.50	0
Mycgr3G102083	2,058	chr_1:1020739–1022796	100	93.75	6.25

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^R-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

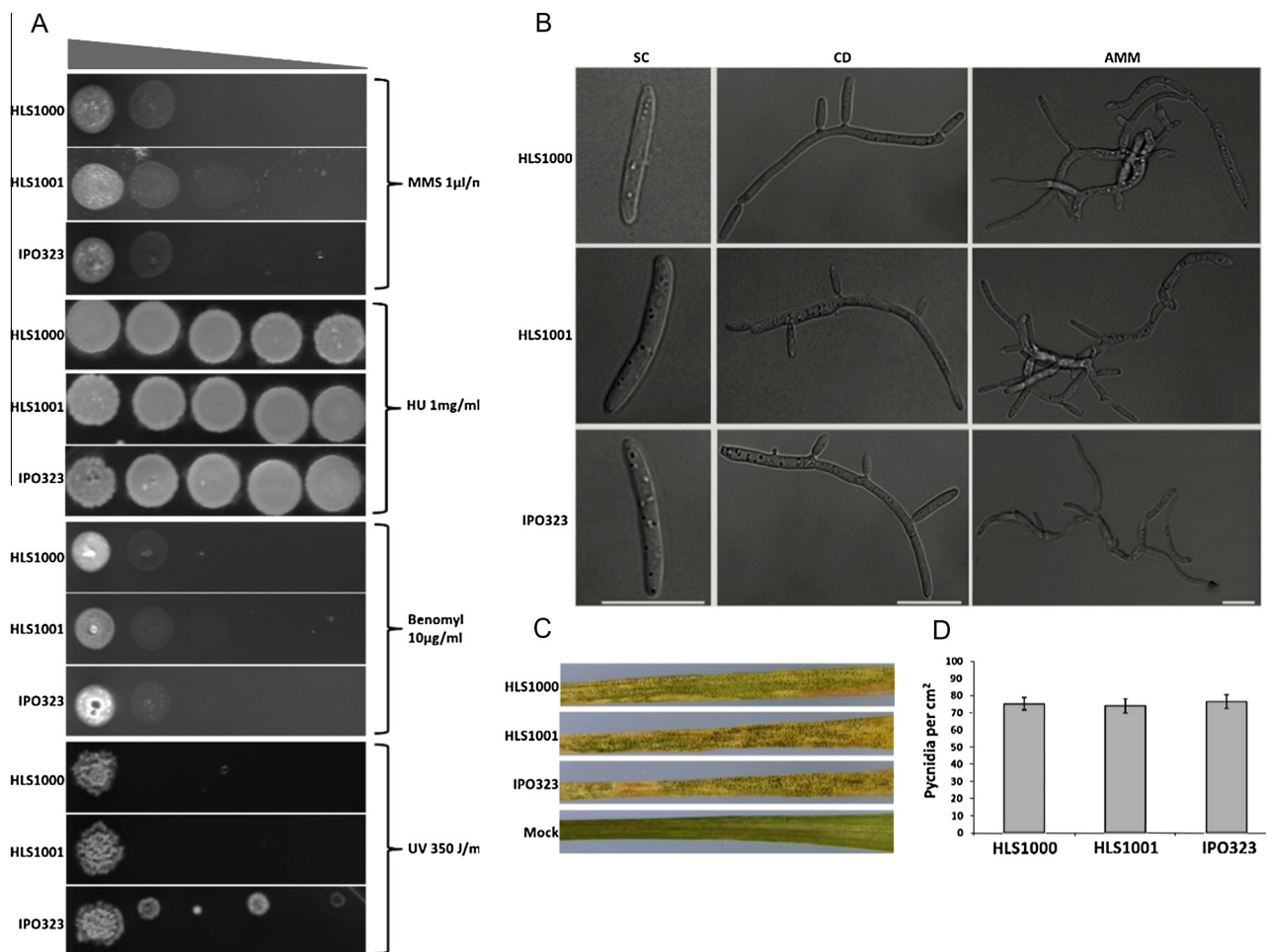


Fig. 3. *Z. tritici ku70* and *ku80* null mutants are essentially wild type in respect of mutagen sensitivity, *in vitro* growth and pathogenicity. (A) *Z. tritici* strains HLS1000 (*ku70* null) and HLS1001 (*ku80* null) are essentially wild-type in their response to methyl methanesulfonate (MMS), benomyl and hydroxyurea (HU), but exhibit mildly increased sensitivity to ultraviolet (UV) radiation as compared to the wild type strain IPO323. Serially diluted cells (10⁸–10⁴ 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 × 10⁷ 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).

transformants 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 6.25% 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 *KU80* 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* β -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 sulphate (MMS), hydroxyurea (HU) or benomyl (Fig. 3A). These compounds result in point mutations 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 *KU80*) 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, HLS1001, HLS1002 and the wild type progenitor strain IPO323. Visual inspection of infection revealed no differences in the initiation of chlorosis at 14 DAI, in appearance of typical black brown pycnidial lesions at 18 DAI and necrosis at 21 DAI on the leaves infected with any strain (Figs. 3C and S3D). Quantification of infection suggested that inactivation of *KU70* or *KU80* did not affect virulence and both *Z. tritici* HLS1000 and HLS1001 were as pathogenic as the wild type strain IPO323 (Fig. 3D). Microscopic observation at 21 DAI revealed secretion of pycnidiospore-bearing cirrhi from the characteristic black-brown pycnidial lesions formed on leaves infected by strains HLS1002 and IPO323 (Fig. S3E, data not shown for strains HLS1000 and HLS1001). The similar progression of infection and specifically the presence of pycnidiospores, the asexual spores required for initiation of multiple STB infection cycles during the growth season (Orton et al., 2011) demonstrate that neither expression of the resistance cassette, nor the deletion of the *Z. tritici* *KU70* or *KU80* genes detectably affect pathogenicity *in planta*.

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 *Z. tritici* HLS1000 and HLS1001 facilitated targeted gene inactivation at higher efficiency (on average >90%) than wild type, for the three different genomic loci that were tested. These results also confirm 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 HR (Rothstein, 1991) in most filamentous fungi the NHEJ pathway mediates DNA damage repair (Villalba et al., 2008; Ninomiya et al., 2004). The

phenotypic screen confirmed that the *in vitro* growth and *in planta* pathogenicity of the *ku70* and *ku80* null strains is comparable to the wild type strain. These results suggest that the *ku70* and *ku80* null strains are an ideal platform for construction of gene deletion mutants in *Z. tritici*. In *N. crassa* and *M. oryzae* the NHEJ mutants have proved to be an excellent platform to dissect novel roles of multiple transcription factors (Colot et al., 2006) and autophagy (Kershaw and Talbot, 2009). *N. crassa* NHEJ mutants also enabled construction of the whole genome deletion library (Collopy et al., 2010) which is an invaluable resource for providing new insights into molecular and cell biology (Fu et al., 2011; Berepiki and Read, 2013). Thus, the *Z. tritici* *ku70* and *ku80* null strains and other tools reported in this study should play an important role in furthering functional analysis in this important pathogen.

4. Conclusion

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 *ku80* 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*.

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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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