A small secreted protein from *Zymoseptoria tritici* interacts with a wheat E3 ubiquitin to promote disease

Sujit Jung Karki[1], Aisling Reilly[1], Binbin Zhou[2], Maurizio Mascarello[1,4] James Burke[1], Fiona Doohan[2], Dimitar Douchkov[3], Patrick Schweizer[3]† and Angela Feechan[1]

[1] *School of Agriculture & Food Science and UCD Earth Institute, University College Dublin, Belfield, Dublin 4, Ireland*

[2]*School of Biology and Environmental Science and UCD Earth Institute, University College Dublin, Belfield, Dublin 4, Ireland*

[3] *Institute of Plant Genetics and Crop Plant Research (IPK), Cytogenetics, Gatersleben, D-06466, Germany*

[4] *Ecology, Evolution and Biodiversity Conservation, Charles Deberiotstraat 8 32, 3000 Leuven, Belgium.*

† In memory of Patrick (author deceased).

Author e-mail addresses:
sujit.karki@ucd.ie
aisling.reilly.2@ucdconnect.ie
binbin.zhou@ucd.ie
maurizio.mascarello@plantentuinmeise.be
Jimmy.Burke@ucd.ie
douchkov@ipk-gatersleben.de
schweiz@ipk-gatersleben.de
fiona.doohan@ucd.ie
angela.feechan@ucd.ie

Corresponding Author: Angela Feechan
angela.feechan@ucd.ie

ABSTRACT

Septoria Tritici Blotch, caused by the ascomycete fungus *Zymoseptoria tritici*, is a major threat to wheat production worldwide. The *Z. tritici* genome encodes many small, secreted proteins (*ZtSSP*) that likely play a key role in the successful colonisation of host tissues. However, few of these *ZtSSP*s have been functionally characterised for their role during infection. In this study, we identified and characterised a small, conserved cysteine-rich secreted effector from *Zymoseptoria tritici* which has homologues in other plant pathogens in the dothideomycetes. *ZtSSP2* was expressed throughout *Z. tritici* infection in wheat with the highest levels observed early during infection. A yeast two-hybrid assay revealed an interaction between ZtSSP2 and wheat E3 ubiquitin ligase in yeast and this was further confirmed *in planta* using bimolecular fluorescence complementation, and co-immunoprecipitation. Down-regulation of this wheat E3 ligase using virus-induced gene silencing, increased the susceptibility of wheat to Septoria tritici blotch (STB). Together these results suggest that TaE3UBQ likely plays a role in plant immunity to defend against *Z. tritici.*

Key words: *Zymoseptoria tritici*, fungal pathogen, disease, wheat, E3 ubiquitin ligase, effector

INTRODUCTION

Plant innate immunity includes the recognition of broadly conserved pathogen-associated molecular patterns (PAMPs), for example fungal chitin, by plant pattern recognition receptors (PRRs). This recognition initiates PAMP-triggered immunity (PTI) to mount a primary defence (Jones and Dangl, 2006). Pathogen effector proteins have evolved to bypass the initial defence response (PTI) resulting in a scenario termed Effector Triggered Susceptibility (ETS). These proteins are typically small, cysteine rich secreted proteins, and are known to manipulate host physiology and interfere with plant immunity (Dou and Zhou, 2012). In return, plants possess Resistance (R) genes which, upon recognition of pathogen effectors, activate Effector Triggered Immunity (ETI) (Jones and Dangl, 2006; Deslandes and Rivas, 2012).

Septoria tritici blotch (STB) caused by *Zymoseptoria tritici* is one of the most prevalent and economically devastating diseases in wheat-growing areas worldwide (Eyal *et al.,* 1985; Fones and Gurr, 2015). As with other fungal pathogens, *Z. tritici* is known to produce a series of small, secreted effector proteins (SSPs) throughout its colonisation of wheat (do Amaral *et al*., 2012; Mirzadi Gohari *et al*., 2015; Rudd *et al*., 2015). Only a handful of *Z. tritici* effectors have been characterised for their role in pathogenesis: *Mg3LysM* and *Mg1LysM* are lysin motif containing effectors that play an important role during the initial symptomless period of *Z tritici* infection (Marshall *et al*., 2011). *Mg3LysM* competes with host chitin receptors by binding fungal chitin fragments (Lee *et al*., 2014). Another *Z. tritici* effector, MgNLP, belongs to the Necrosis and Ethylene-Inducing Peptide 1 (NEP1)-like (NLP) family of proteins. It can induce cell death in *Arabidopsis*, but not in wheat (Motteram *et al*., 2009). In addition, *Z. tritici* secretes two necrosis inducing protein effectors (ZtNIP1 and ZtNIP2) that induce cell death and chlorosis in some wheat cultivars (M' Barek *et al*., 2015).

Recently, several *Z. tritici* candidate effectors were found to induce a cell death phenotype when expressed in the non-host plant *Nicotiana benthamiana* (Kettles *et al*., 2017). One of these candidate effectors was subsequently identified as a novel fungal PAMP, termed Cell Death-Inducing 1 (ZtCDI1) (Franco-Orozco *et al*., 2017).

Similarly, another candidate (Zt-6) was characterized as a secreted ribonuclease which possess cytotoxic activity against other microbes as well as plants (Kettles *et al*., 2018). Additionally, avirulence effectors, namely Avrstb6 (Kema *et al*., 2018; Zhong *et al*., 2017) and Avr3D1 (Meile *et al*., 2018), are known to be recognized by specific wheat cultivars. Recently, *Z. tritici* small, secreted proteins were found to interact with wheat Septoria Responsive Taxonomically Restricted Genes namely, TaSRTRG6 and TaSRTRG7 implicated in STB disease (Brennan *et al.,* 2020). However, the function of these TRGs is unknown. While some *Z. tritici* candidate effectors have been identified, the molecular targets in wheat and their role during infection remain largely unknown. Therefore, there is a need to identify the wheat host proteins targeted by these *Z. tritici* effectors.

In this study, we selected a conserved effector candidate (ZtSSP2) similarly expressed in three different *Z. tritici* isolates. *ZtSSP2* is a small, secreted protein (22KDa) with ten cysteine residues that appears to be conserved within *Z. tritici* isolates and other dothideomycete fungus. Furthermore, we showed that *ZtSSP2* physically interacts with wheat E3 ubiquitin ligase (*TaE3UBQ*) and that virus-induced gene silencing (VIGS) of TaE3UBQ resulted in increased *Z. tritici* susceptibility. This study provides insights in to how a *Zymoseptoria tritici* effector likely targets a host ubiquitin system to aid successful colonization.

MATERIALS AND METHODS

Plant material, fungal strains, and growth conditions.

Nicotiana benthamiana and wheat (*Triticum aestivum*) cv. Remus, Kanzler and Longbow were used in this study. The cv. Remus and cv. Kanzler are moderately susceptible to *Z. tritici* (Váry *et al*., 2015; Brady, 1983) while cv. Longbow is susceptible to *Z. tritici* (Brading *et al*., 2002). *N. benthamiana* plants were grown growth chambers at 16-hour light at 22°C / 8-hour dark at 18°C with 7500-8200 lux, RH 70% ± 5% throughout the experiments and 4-6-week *N. benthamiana* plants were used for localisation studies.

Wheat seeds (cv. Remus, cv. Longbow) were surface-sterilised and incubated for 3 days at 4ºC for seed stratification and then incubated for 4 days at room temperature without illumination to allow germination. Germinated seeds were transferred into plastic pots containing John Innes Compost No. 2 (Westland Horticulture, UK) and grown in a growth chamber at 16 hours day/8-hour night photoperiod at 13,000 lux, RH 80% ± 5% at 19°C/12^{o}C. For biolistic studies, wheat cv. Kanzler seedlings were grown in a growth chamber at 16 hours day/8-hour night photoperiod at 15,000 lux, RH 60% ± 5% at 20°C in pots containing IPK soil substrate.

The *Z. tritici* isolate IPO323 (Kema and van Silfhout, 1997), Irish isolates 560.11 (Lynch *et al*., 2016) and 553.11 were used to infect the susceptible wheat cv. Remus and Longbow. Both 560.11 and 553.11 were isolated from the wheat cv. Alchemy in 2011 from South Wexford, Ireland (S. Kildea pers. comm). Prior to use, isolates were cultured on potato dextrose agar (PDA) and grown at 20^{o}C for approximately 5-7 days. Fourteen-day old wheat seedlings were used for inoculation. For the disease assay we used 10^6 cfu/ml spore suspension as inoculum (Fones *et al.,* 2017; Shetty *et al.,* 2003; Zhong *et al.,* 2017) to assess pycnidia and necrosis coverage following VIGS of TaE3UBQ. Fungal spores from PDA cultures were harvested and spore concentration was adjusted to 1 x 10^6 ml^{-1} in water containing 0.02% Tween 20. Spore suspensions (5 ml per plant) were sprayed to runoff (5 ml) per wheat plant using hand-held spray bottles. Control plants were sprayed with 5 ml of 0.02% Tween 20 solution. Inoculated plants were covered with polythene bags to ensure high humidity and removed after 72 h.

Effector candidate selection

The publicly available secretome dataset from do Amaral *et al*., 2012 which is based on the IPO323 reference genome (Goodwin et al., 2011), was mined to identify *Z. tritici* small, secreted proteins (*ZtSSPs*). 262 candidate genes with EST support were screened based on small size (50 to 315 amino acids), resulting in 102 SSP's. These were sorted based on the number of cysteine residues which resulted in 90 SSP's with multiple cysteines (Fig. S1). The amino acid sequence was then used to predict effector properties and any apoplastic localisation using EffectorP & ApoplasticP (Table S1) (Sperschneider *et al*., 2016, 2018). These were analysed using NCBI CDD (Conserved Domain database) to update the prediction of any conserved domains. Candidate effector proteins were analysed using BLASTP (cut-off value ≥ 50% Identity and e-value ≤ 0.01) to search for homologues in other plant pathogenic fungal species (Table S2). Finally, those that were unannotated (Table S1) and with a potential homologue in other plant pathogenic fungi were selected. Of these, 17 non-annotated *ZtSSP*s were analysed for expression among three *Z. tritici* isolates (IPO323, 553.11 and 560.11) at 7dpi. (Table S3). *Mycgr3G105265* (*ZtSSP2*) was selected for further study based on these criteria and the similar expression levels across all three isolates.

Transcriptomics analysis

Leaves (cv. Longbow) infected with the *Z. tritici* isolates IPO323, 553.11 and 560.11 from four independent replicates were collected at 7 dpi. RNA from each sample was extracted at room temperature using the RNeasy Plant Mini Kit (QIAGEN), purified from DNA contamination using DNAse I (Sigma Aldrich) and stored at 20 ºC. RNA quality control was assessed by Agilent 2100 bioanalyzer. RNA was extracted from two leaves from each of two seedlings infected with each isolate over four independent experiments (n = 16), pooled and sent for RNA sequencing on an Illumina HiSeq 2000 (paired-end 100 bp reads) at the 250 Bejing Genome Institute (BGI) (Hong Kong).

Expression analysis was performed by General Bioinformatics (Reading, UK). The reference genome for *Z. tritici* was from the MG2 assembly from ENSEMBL Fungi release. The reference genome for *Triticum aestivum* was from the IWGSC1+popseq

assembly from ENSEMBL Plants release. The quality control of raw reads was assessed with FastQC v0.11.5 (Andrews, 2010). Reads were trimmed to remove contaminating adapter sequences and poor-quality bases at the beginning of the reads using Trimommatic (Bolger *et al.,* 2014). Clean reads generated were 185,725,832 for IPO323 isolate infected leaf samples, 187,073,058 for 553.11 isolate infected leaf samples and 183,036,662 for the 560.11 isolate infected leaf sample. Unfiltered reads were aligned to the *Z. tritici* genome using Tophat v2.1.1 aligner (Kim *et al.,* 2013). BAM files for reads mapped to the *Z. tritici* genome were converted to SAM files and sorted for further analysis using Samtools v 1.3 (Li *et al*., 2009). Then, reads were counted using the htseq-count script of the HTSeq v 0.6.0 (Anders *et al.,* 2015). Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values for each sample were calculated using Cufflink package (Trapnell *et al.,* 2010). Genes with a total read count lower than 1 were filtered out. Counts were normalised using TMM (Robinson *et al.,* 2010) and the common dispersion BCV (square-root-dispersion) was set at 0.4. Pairwise comparison between datasets were made using the exact test (Robinson *et al.,* 2008). Filtered reads were aligned to the *T. aestivum* genome using Tophat v2.1.1. The BAM file containing the unmapped reads was converted back to FastQ format using the bam2fastx utility of Tophat (Kim *et al.,* 2013). The alignment to the *Z. tritici* genome and subsequent analysis were performed as described for the unfiltered reads.

Amplification and Cloning of *ZtSSP*2

ZtSSP2 was amplified from wheat (cv. Remus) infected with *Z tritici* isolate 560.11 c-DNA with and without the signal peptide using Phusion High Fidelity Polymerase (New England Biolabs) and primers flanked with gateway adapter sequence (Table S4). AttB-flanked PCR products were purified using QIA quick PCR Purification Kit (Qiagen) and cloned into pDONR207 (Invitrogen) using BP clonase II enzyme mix (Thermo Fisher Scientific) and subsequently cloned into the binary vector pEARLYGATE 101 (pEG101) (Earley *et al.,* 2006) using LR clonase II enzyme. Entry clones were sequence verified by sequencing (Macrogen Europe) before LR reaction. All other destination vectors are described separately.

Validation of protein secretion using a yeast sucrose secretion system

A gateway compatible vector (pGADT7) for yeast secretion assay and *suc2* yeast mutant (strain SEY6210) was utilised (Brennan *et al.,* 2020). Briefly, the invertase (*SUC2)* gene with and without signal peptide was amplified from the yeast strain BY4741 with a linker (Kex2 site) added between the gateway reading frame and *SUC2* gene. This construct was ligated into pGADT7 vector and verified by sequencing. Candidate *ZtSSP2 and ΔSP-ZtSSP2* was cloned into the yeast secretion vector in frame with N terminus of *SUC* gene and transformed into *suc2* yeast mutant. Transformants were PCR validated and selected on a synthetic dropout medium (minus Trp and leu) with sucrose as a sole carbon source. Yeast spotting was performed with dilution of 10^{-1}, 10^{-2} and 10^{-3} respectively. The experiment was repeated three times independently with three replicates per experiment.

Phylogenetic analysis of ZtSSP2

BLASTp search was performed using the NCBI (National Centre for Biotechnology Information) BLAST service (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and uniprot blast (http://www.uniprot.org/blast/). Sequences of the 20 closest homologues of ZtSSP2 (Table S5) were aligned using ClustalW, and a phylogenetic tree was constructed using the maximum likelihood (ML) method with 1000 bootstrap replicates in Mega7 (Kumar *et al*., 2016).

RNA extraction and quantitative RT-PCR

100 mg of infected leaves (cv. Remus) per sample was collected at different days post infection (dpi), frozen in liquid nitrogen and stored at -80 ºC. Total RNA was extracted from *Z. tritici* infected wheat leaves using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. RNA was then subjected to on-column DNase treatment (Sigma). Quantification of total RNA was carried out using a Nanodrop ND-1000 spectrophotometer. Reverse transcription of 1-2µg RNA for cDNA synthesis was carried out using Omniscript RT Kit (Qiagen).

Real-Time quantitative PCR was carried out in 12.5 µl reactions including 1.25 µl of a 1:5 (v/v) dilution of cDNA, 0.2 µM of primers, and 1× SYBR Premix Ex Taq (Tli RNase H plus, RR420A; Takara). PCR conditions were as follows: 1 cycle of 1 min at 95°C; 40 cycles of 5 s at 95°C and 20 s at 60°C; and a final cycle of 1 min at 95°C, 30s, at 55°C, and 30 s at 95°C for the dissociation curve. For *ZtSSP2* expression, RNA was extracted from the 3rd leaf of wheat seedlings and from three individual leaves from different seedlings per time point per replicate. Three independent experiments were performed. qPCR was performed using QuantStudio 7 Flex Real-Time PCR system (Applied Biosystems) and the relative gene expression was calculated as $2^{-(\text{Ct target gene - Ct housekeeping gene})}$ as previously described (Livak and Schmittgen, 2001). *Z. tritici tubulin* was used as housekeeping gene control for the ZtSSP2 time course. While for VIGS, Ct housekeeping gene = geometric mean (Ct *Tacdc48*: Ct *Taelf4E*) of two wheat reference genes; Cell division control protein 48 (*TaCDC48*) and Eukaryotic Initiation factor 4E (*TaeIF4E)* (Lee *et al*., 2014).

Single-cell death assay in wheat

The cell death assay in wheat was performed as previously described (Pliego *et al*., 2013). Briefly, seven leaves of 7-day old wheat (approx. 8 cm in length) cv. Kanzler was co-bombarded (PDS-1000/He System, Bio-Rad) with 7µg of pEG101: (ΔSP) *ZtSSP2* (over-expression), 7µg pUbiGUS (β-glucuronidase reporter for transformation efficiency) and 7µg of the *B-Peru/C1*-expression plasmid pBC17 (Schweizer *et al*., 2000) for inducing anthocyanin production (as a marker for live cells). Four days post bombardment, cells accumulating anthocyanin were counted and leaves were then stained with 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) solution overnight at 37°C. Leaves were destained with trichloroacetic acid (Douchkov *et al*., 2005). The number of cells with visible GUS stain was counted and the relative number of anthocyanin producing cells was calculated as the ratio of anthocyanin accumulated cells to the number of β-glucuronidase (GUS) expressing cells. The experiment was repeated four times independently and seven leaves counted per replicate.

Yeast two-hybrid analysis

The c-DNA library was comprised of leaf three of wheat seedlings cv. Stigg and Longbow which were infected with a mixture of *Z. tritici* isolates (IPO323, 560.11 and Cork cordiale 4) (Brennan *et al*., 2020; Kema *et al*., 1997; Lynch *et al*., 2016), collected at various time points (1, 2, 4, 6, 8, 10, 12 dpi) then pooled together for RNA extraction. Briefly, the c-DNA of (ΔSP) *ZtSSP2* was cloned into pB27 vector as an N-LexA-bait-C fusion to LexA. The construct encoding ZtSSP2 was used as bait to screen the c-DNA library of wheat leaves inoculated with *Z. tritici*.

The initial yeast two-hybrid screening was performed by Hybrigenics Services, S.A.S. (http://www.hybrigenics-services.com). A total of 76.4 million clones were screened and 73 positive clones were processed following selection on selective medium lacking Trp, Leu, and His supplemented with 0.5mM 3AT. The prey fragments from positive clones were amplified and sequenced. The putative high confidence interactors are listed in Table S6. These sequences were used to identify corresponding proteins in the NCBI GenBank database including TaE3UBQ. For analysis of a specific interaction, CDS of *TaE3UBQ*, *TaE3UBQ*$_{126\text{-}219}$, barley homologue of E3UBQ; HvUBQ and (ΔSP) ZtSSP2 and the *Ramularia collo-cygni* homologue of ZtSSP2; RcSSP2 was cloned into the vector pDONR207 using Gateway cloning technology. They were then recombined into bait and prey vectors derived from pGADT7 and pGBKT7 plasmids (Clontech, USA). Analysis of protein-protein interactions was performed using the Gal4 two-hybrid assay as described in Perochon *et al*., 2010. As a negative control to ensure specific interactions with ZtSSP2, another small secreted effector candidate (ΔSP) Zt-10 was used (Kettles *et al*., 2017) while the positive control included TaSSP6 and Zt-06 (Zhou *et al.,* 2020).

Agrobacterium mediated transient expression

A. tumefaciens strain GV3101 was transformed by electroporation with effector constructs and grown for 48 hrs at 28°C 220rpm in LB medium with the antibiotic gentamicin (25mg/ml) and kanamycin (50mg/ml). Transformed cells were harvested by centrifugation and suspended in infiltration buffer (10mM $MgCl_2$, 10mM 2-(N-mopholino) ethanesulfonic acid (MES) pH 5.6 and acetosyringone 150µM) at an

absorbance 600nm (OD_{600}) of 0.5. The bacterial suspension was left at room temperature for 2hr before infiltration into 4-6-week-old *N. benthamiana* on the abaxial side of the leaves using a 1ml needleless syringe.

In planta validation of protein-protein interaction

For *in planta* analysis of the interaction between (ΔSP) ZtSSP2 and TaE3UBQ the coding sequences were cloned in the gateway vector pDONR207 (Invitrogen, USA) and subsequently cloned into the bimolecular fluorescence complementation (BiFC) vectors pDEST-GW VYCE, pDEST-VYCE GW, pDEST-GW VYNE and pDEST-VYNE GW (Gehl *et al*., 2009). This resulted in constructs where proteins were fused at either the N or C terminal to the YFP C-terminal (YFP^C) or N-terminal fragment (YFP^N). For localisation of TaE3UBQ, LR reaction was performed with pGWB406 (Nakagawa *et al*., 2007). Vectors were transformed into *A. tumefaciens* strain GV3101 by electroporation. Transformants containing the plasmids were selected on LB agar plates containing 10 $\mu g ml^{-1}$ rifampicin, 20 $\mu g ml^{-1}$ gentamicin and 50 $\mu g ml^{-1}$ kanamycin. A mix of Agrobacterium transformants was prepared: OD_{600} = 0.5 and 0.5 and 0.1 of YFPC construct, YFPN and P19 silencing construct, respectively. This mix was syringe-infiltrated into leaf epidermal cells of 3-4-week-old *N. benthamiana*, by making a small injury into the leaf and pressure infiltrating. For TaE3UBQ localisation, MG132 (100 µm) was infiltrated into leaves for 6h before analysis to prevent protein degradation. Images were analysed using a confocal laser scanning microscope (Olympus fluoview FV1000). GFP and YFP excitation was performed at 515 nm and emission detected in 530 to 630 nm range. These experiments were repeated at least twice independently, and each experiment included three leaves, each from an individual plant.

Co-immunoprecipitation (Co-IP) Assay

The protein construct was transiently overexpressed in *N*. *benthamiana* leaves using agro-infiltration. Leaf samples were collected at 48hr post infiltration, TaE3UBQ and GFP samples were infiltrated with MG132 (100 µm) 6h before collection. Proteins were extracted using GTEN buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM

EDTA, 10% glycerol 0.1% Tween 20) with 2% W/V polyvinylpolypyrrolidone (PVPP), 10 mM dithiothreitol (DTT), and a protease inhibitor cocktail (Sigma). Samples were incubated for 15 min in lysis buffer (4°C). Lysate was centrifuged at 10,000g for 10 min and 250 µl of supernatant subjected to Co-IP with GFP-Trap®-M magnetic beads (Chromotek, Germany) for affinity binding of GFP-fused proteins at 4°C for 4 hours. The beads were washed three times with 500 µl of extraction buffer. Protein bound to magnetic beads was boiled for 10 min for elution. Eluted proteins and crude proteins (input) were detected by western blotting. Immunoblotting of the proteins on the PVDF membrane were detected using the corresponding anti-HA (1:1000; Roche) and anti-GFP (1:5000; Invitrogen) antibody.

BSMV mediated gene silencing (VIGS)

The barley stripe mosaic virus (BSMV)-derived VIGS vectors used in this study consisted of the wild type BSMV ND18 α, β, γ tripartite genome (Holzberg *et al*., 2002; Scofield *et al*., 2005). A BSMV γ vector construct containing a 185 bp-fragment of the barley phytoene desaturase gene (BSMV: PDS) was used as positive control for VIGS, as previously described (Scofield *et al*., 2005) (Fig. S2). Two independent, non-overlapping gene constructs (UBQV1 and UBQV2) were used for gene silencing (Fig. S3). Both gene fragments were PCR-amplified and chosen to target *TaE3UBQ-1D* and its homoalleles (*TaE3UBQ-1A* and *TaE3UBQ-1B)*. The specificity and silencing efficiency were evaluated by BLASTn and using SGN VIGS tool with *T. aestivum IWGSC2* as a database (Fernandez-Pozo *et al*., 2015). PCR-amplified fragments were ligated in the antisense orientation into *Not*I/*Pac*1-digested BSMV γ vector pSL038-1 (Scofield *et al*., 2005). Construct authenticity was verified by sequencing.

Vectors containing the BSMV α, γ genomes and the γ genome vectors containing either BSMV: UBQV1, BSMV: UBQV2, or BSMV: PDS were linearized with *Mlu*I. The BSMV β genome was linearized with *Spe*I. Capped *in vitro* transcripts were prepared from the linearized plasmids using the mMessage mMachine T7 in vitro transcription kit (AM1344, Ambion) following the manufacturer's protocol. RNA quantity and quality were evaluated using the ND-1000 spectrophotometer (NanoDrop,Thermo Fisher Scientific, United States) measurement. Capped BSMV transcripts (1:1:1)

with 1X FES buffer were rub-inoculated onto the second leaf of the two-leaf stage of the wheat cultivar Longbow. BSMV: PDS was used as positive control, whereas BSMV γ and 1X FES buffer was used as negative control. Fourteen days after virus inoculation, the third and fourth leaves of virus inoculated wheat seedlings (12 plants per treatment per trial) were infected with *Z. tritici* (560.11). The third leaves were collected at 10 dpi and RNA extracted individually from each of the leaves per treatment. The fourth leaves were used for STB disease symptom phenotyping at 21 days post inoculation (10 leaves per treatment, 3 independent trials). Seven leaves per treatment per replicate were submerged in 10 ml deionised water for 1 hour and vortexed to collect spores and 10 ul counted using a haemocytometer.

Statistical Analysis

Statistical analysis of the data was carried out using the R statistical software (R Core Team, 2016). All data from the studies were checked for normal distribution and when necessary, variances were stabilized using Box-cox transformation. A generalised linear model was used to test the data and significant differences were determined using the Tukey test at $P < 0.05$. For analysis of VIGS data (phenotyping and spore counts), data were fitted to a generalised linear mixed model with binomial distribution to account for over-dispersion and zero-inflation. Significance of differences between treatments was assessed using the Tukey's HSD.

RESULTS

1. ZtSSP2 is a conserved small, secreted protein candidate

The publicly available secretome dataset from do Amaral *et al.,* 2012 was mined to identify *Z. tritici* small, secreted proteins (*ZtSSPs*). 262 candidate genes with expressed sequence tag (EST) support were screened based on small size (50 to 315 amino acids) resulting in 102 *ZtSSPs*. These proteins were then sorted based on number of cysteine residues (≥1) which resulted in 90 *ZtSSPs* (Fig. S1). The amino acid sequence was then used to predict effector properties and any apoplastic localisation using EffectorP & ApoplasticP (Table S1). Further analysis of these candidates showed that 52% were predicted to be effector proteins while, 81% were predicted to be apoplastic proteins (Table S1) (Sperschneider *et al*., 2016, 2018). The 90 candidate effector proteins were analysed using BLASTP (cut off value as ≥ 50% identity and E-value ≤ 0.01) to search for homologues. Fifty-nine of these ZtSSP candidates had a homologue in another plant-pathogenic fungal species (Table S2 and S7). Of the fifty-nine ZtSSPs thirteen were found to have a homologue in *Zymoseptoria brevis* only (Table S7). Forty-three ZtSSPs had a homologue in in *Z. brevis* as well as another plant pathogen while three had a homologue in a different plant pathogenic species other than Z. *brevis* (Table S2). Of the forty-three, seventeen were non-annotated proteins and twenty-six candidates had annotated protein domains. We examined the expression of these seventeen conserved non-annotated effector candidates across three *Z. tritici* isolates at 7 dpi. The expression of Mycgr*3G105265 (ZtSSP2)* was similar (FKPM 148, 126 and 177) across all three isolates (IPO323, 553.11 and 560.11 respectively) compared to other *ZtSSP* candidates (Table S3). We selected the effector candidate ZtSSP2 for functional characterisation as we reasoned that the conservation across different plant pathogenic species (Table S2:S5) and the similar levels of expression across three different *Z. tritici* isolates (Table S3) may indicate a conserved core effector.

ZtSSP2 is predicted to have an N-terminus signal peptide (0.9986 likelihood, SignalP4.1) (Nielsen et al., 2017). To validate this, we tested the secretion of ZtSSP2 using a yeast secretion assay (Fig 1a; Brennan *et al.,* 2020). The full length ZtSSP2 protein could complement the suc2 knock out yeast strain allowing it to grow

in selection media containing sucrose as a sole source of carbon (Fig. 1b). These characteristics suggest ZtSSP2 is a conserved secreted effector protein.

2. Homologues of ZtSSP2 are conserved in the Dothideomycete fungi.

For ZtSSP2 we found additional potential homologues in other dothideomycetes. All the potential homologues of ZtSSP2 identified within the plant pathogenic *Mycosphaerellaceae* family were of similar size, possessed an N-terminal signal peptide and contained ten conserved cysteine residues (Fig. 2a). For example, the homologue from the conifer infecting fungi *Dothistroma septosporum* shares 53.57% sequence similarity, that from the barley pathogen *Ramularia collo-cygni* is 51.67% identical to ZtSSP2, and that from the banana pathogen *Mycosphaerella fijiensis* shares 51% sequence identity. The majority of homologues (13) were from plant pathogens but other homologues were from two eurotiomycetes which are human pathogens (Fig. 2b) (Table S5). There was also an outgroup of dothidoemycetes which were non-pathogenic (Fig. 2b).

3. *ZtSSP* expression during STB infection in wheat

Pathogen effector genes are known to be induced transcriptionally during infection of the host plant (Stergiopoulos and de Wit, 2009). We performed a quantitative reverse transcription PCR (qRT-PCR) to determine the expression of *ZtSSP2* during infection. The *Z. tritici* isolate 560.11 (Lynch *et al*., 2016) was used to infect wheat (cv. Remus) and the expression of *ZtSSP2* determined over 2, 4, 8, 10 dpi (representing biotrophic stage), 14, 21 dpi (necrotrophic stage) compared to the uninfected control (Fig. 3). Based on expression analysis, *ZtSSP2* was expressed from 2 dpi through to 21 dpi. The expression was significantly higher at 2 dpi compared to all other time points suggesting potential role early in biotrophy. There was a significant dip in expression at 14 dpi compared to 2 and 21 dpi (Fig.3).

4*. ZtSSP* candidates did not induce cell death in wheat

We used a transient leaf expression system by Pliego *et al*., 2013, to assess whether ZtSSP2 can induce cell death. In this system transient expression of the maize

transcription factor *B-Peru* and *C1* leads to accumulation of anthocyanin only in intact vacuoles of viable cells and can therefore be used as a cell death marker (Schweizer *et al*., 2000). We co-bombarded pEG101: (ΔSP) *ZtSSP2* overexpression construct with pUbiGUS (cell death insensitive transformation marker) and anthocyanin expression plasmid pBC17 into wheat leaves. Co-bombardment of pBC17 with pUbiGUS and the vector pEG101 was used as a negative control while, *Zt-6* previously reported to induce cell death in wheat (Kettles *et al*., 2018) was used as a positive control, respectively. The number of cells accumulating anthocyanin (Fig. 4a) was counted and then leaves were subsequently stained for GUS activity (Fig. 4b). The number of cells stained with GUS was also counted. The ratio of anthocyanin to GUS cells was used as an estimate of cell death. The empty vector control (pEG101) resulted in the average ratio of 1.27 (Fig. 4c), while the positive control (pEG101: *Zt-6*) showed a reduced ratio of 0.3 ($P < 0.05$). The anthocyanin to GUS ratio obtained from bombardment of ZtSSP2 was not significantly different from the control suggesting no cell death activation by this effector candidate in wheat.

5. Candidate ZtSSP2 interacts with wheat ubiquitin ligase *in vitro* and *in planta*

We performed a yeast two-hybrid screen to determine if ZtSSP2 could interact with wheat host components. A cDNA expression library generated from *Z. tritici* infected wheat leaves was screened using ZtSSP2 as bait. A wheat cDNA clone encoding the extracellular region (amino acids 126-219) of a C3H2C3-type RING E3 ubiquitin ligase protein (TaE3UBQ) was identified. Pathogen effectors have been reported to target the host ubiquitin system to manipulate host defence (Park *et al*. 2012). To investigate if ZtSSP2 interacts with the wheat E3 ligase (TaE3UBQ) and thereby manipulates the wheat host ubiquitin proteasome system this protein-protein interaction was tested in yeast (*Saccharomyces cerevisiae*) using a galactose-responsive transcription factor GAL4-based yeast two-hybrid system (Fig. 5a). We cloned TaE3UBQ and $TaE3UBQ_{126\text{-}219}$ from wheat cDNA and found that (ΔSP)ZtSSP2 interacted with $TaE3UBQ_{126\text{-}219}$ but not with the full-length protein in yeast (Fig. 5a). TaE3UBQ homologue HvE3UBQ was found in barley, and the ZtSSP2 homologue RcSSP2 found in the barley pathogen *Ramularia collo-cygni* (Fig. 6). Therefore, this interaction was also tested (Fig. S4). However, no positive

interaction between (ΔSP)RcSSP2 and HvE3UBQ was observed. When RcSSP2 was replaced with (ΔSP)ZtSSP2 we found that $HvE3UBQ_{126\text{-}219}$ also interacts with ZtSSP2 in yeast (Fig. S4).

Additionally, the interaction between (ΔSP)ZtSSP2 and TaE3UBQ was investigated *in planta* using the BiFC system (Gehl *et al*., 2009). TaE3UBQ and (ΔSP)ZtSSP2 were fused at the N terminus and C-terminus of YFP respectively. The resulting constructs were co-expressed using *Agrobacterium* infiltration in *N. benthamiana* leaves. A strong YFP signal was observed, when YFP^N–TaE3UBQ was co-infiltrated with YFP^C- (ΔSP)ZtSSP2 (Fig. 5b). To test if the interaction of TaE3UBQ was specific to ZtSSP2, co-infiltration with another putative *Z. tritici* effector; YFP^C- (ΔSP)Zt-10 was performed. No YFP fluorescence was observed with this construct suggesting no interaction of TaE3UBQ with candidate (ΔSP)Zt-10. Cnx6 homodimers were used as positive control (Gehl *et al*., 2009).

We used co-immunoprecipitation (Co-IP) to further validate the interaction of TaE3UBQ with ZtSSP2. The HA-tagged TaE3UBQ was co-expressed with a GFP fusion of ZtSSP2 in *N. benthamiana* and subjected to Co-IP assay using GFP-Trap®-M magnetic beads. Western blot analysis showed that only HA-tagged TaE3UBQ was co-immunoprecipitated on GFP-Trap®-M beads and specifically detected with the anti-HA antibody in presence of GFP-ZtSSP2, but not in the GFP control (Fig. 5c). Taken together, these results reveal that ZtSSP2 interacts with TaE3UBQ *in vitro* and *in planta*.

6. Wheat ubiquitin represents is a C3H2C3 ring finger E3 protein ligase with a transmembrane helix.

The full-length cDNA of TaE3UBQ (TraesCS1D02G119700) was obtained by comparing the clone sequence with EnsemblPlants IWGSC database. BLASTp showed that TaE3UBQ has two additional homeologs in wheat namely: TraesCS1A02G118800 and TraesCS1B02G138300 sharing 99.3 and 97% similarity with its 1D variant respectively. The TaE3UBQ ORF encodes a RING finger protein of 420 amino acids, with a theoretical pI value of 6.06 and a deduced molecular mass of 46.2 kDa (Fig. 6a). Protein sequence analysis using NCBI CDD (Marchler-Bauer *et al*., 2017) and MemBrain 3.1 (Yin *et al*., 2018) programs showed a C-

terminus C3H2C3 Zinc-finger domain with transmembrane helix and extracellular loop region (Fig. 6a, 6b) (Fig. S5). Alignment of TaE3UBQ revealed that the RING finger domain is conserved among various plant species (Fig. 6b). We performed localisation of GFP-tagged TaE3UBQ in *N. benthamiana* leaves. The fluorescent signal from *GFP-TaE3UBQ* was predominantly localised at the cell periphery similar to the signal obtained by expression of a plasma membrane marker pm-yk (Nelson *et al*., 2007), whereas the GFP control was distributed throughout the cell including the nucleus (Fig. 6c).

7. Silencing *TaE3UBQ* enhances wheat susceptibility to *Z. tritici*

We examined the expression of *TaE3UBQ* homeologs using expVIP (expression Visualization and Integration Platform) (Borrill *et al*., 2016). All three wheat homeologs have a similar expression profile and levels were not significantly different between mock and *Z. tritici* treatment (Fig. S6). Virus-induced gene silencing was used to determine the role of *TaE3UBQ* during the interaction of *Z. tritici* with wheat (cv. Longbow) (Fig. 7). Two independent non-overlapping constructs (UBQ_V1, UBQ_V2) were used to target all three *TaE3UBQ* homeologs (Fig. S3). Expression of three homeologs was measured using a primer pair in a conserved region (Fig. S3). qRT-PCR analysis of gene silenced leaf tissue (BSMV:UBQ_1 and BSMV:UBQ_2) showed that, compared to the control (BSMV:00) plants, *TaE3UBQ* expression (all homeologs) was significantly reduced by 53-70% in mock inoculated leaves and 59-76% in *Z. tritici* inoculated leaves of the wheat cv. Longbow (Fig. 7a). Silencing using construct UBQ_V1 showed higher efficiency than construct UBQ_V2. Phenotypic assessment of wheat leaves at 21 days post *Z. tritici* infection revealed that BSMV:UBQ_V1 and BSMV:UBQ_V2 leaves had significantly increased disease coverage as represented with higher necrosis and pycnidia coverage compared to control leaves (BSMV:00), (Fig. 7b,7c). This was reflected by the significantly higher *Z. tritici* spore numbers found in BSMV: UBQ_V1 and BSMV: UBQ_V2 treated leaves compared to the BSMV:00 control (Fig. 7d).

DISCUSSION

The major component of the filamentous plant pathogens secretome are often small, secreted cysteine rich proteins (SSPs) (Stergiopoulos and de Wit, 2009). These SSPs are small (300aa) with cysteine residues and a secretion signal at the N-terminus. SSPs from plant pathogens play a key role in subverting host plant immunity and facilitating colonisation (Hogenhout *et al.,* 2009; Rafiqi *et al.,* 2012). Therefore, understanding how pathogen SSPs function in a host plant and the potential host targets is key for complete understanding of molecular mechanism of pathogenicity and disease.

In this study, we characterise a small secreted protein ZtSSP2, as an effector candidate, from *Z. tritici* which is conserved across the dothideomycetes. Fungal pathogen effectors are secreted in host, have diverse functions and are differentially regulated throughout infection (Chen *et al*., 2013). ZtSSP2 was functionally secreted using a yeast secretion system suggesting it is a potential pathogen secreted effector candidate protein (Fig. 1).

Z. tritici has a long latent/biotrophic phase prior to necrotrophy and has been described as a hemibiotroph (Rudd *et al.,* 2015) or a latent necrotroph (Sánchez-Vallet *et. al,* 2015). During the latent phase which lasts for 7-10 days *Z. tritici* may be endophytic (Sánchez-Vallet *et. al.,* 2015) or even epiphytic (Fones *et al.,* 2017). *ZtSSP2* expression levels were highest at 2 dpi during the latent/biotrophic phase of the pathogen before dipping at 14 dpi following the switch to the necrotrophic phase which takes place from around 10 dpi (Rudd *et al.,* 2015). A biphasic expression pattern was also reported for *ZtSSP2* previously (Mirzadi Gohari *et al.,* 2015).

To test a possible role for ZtSSP2 in cell death and potentially the necrotrophic lifestyle of *Z. tritici*, we utilized a biolistic approach with an anthocyanin marker for cell death in wheat. Our results showed that ZtSSP2 does not induce cell death in wheat leaves. Ubiquitin E3 ligases can act as either positive or negative regulators of plant immunity controlling the degradation of different protein substrates (McLellan et al., 2020). In rice the RING E3 ligases, APIP6 and APIP10 are positive regulators of PTI as well as targets of the *Magnaporthe oryzae* effector AvirPiz-t. When either APIP6 or APIP10 are silenced PTI is compromised including impaired reactive oxygen species production and defence gene induction (Park *et al.,* 2016). The reactive oxygen species, hydrogen peroxide is known to restrict *Z. tritici* growth

during the latent/biotrophic phase (Shetty *et al.,* 2003). It is conceivable that TaE3UBQ plays a similar role in wheat positively regulating PTI. Overexpression of *ZtSSP2 in planta* may promote interaction with TaE3UBQ and compromise PTI which would not induce cell death in agreement with our results (Fig. 4). Silencing TaE3UBQ would also compromise PTI. This is in line with the increased necrosis and pycnidia formation observed with VIGS of TaE3UBQ (Fig. 7). Thus, ZtSSP2, may interact with TaE3UBQ to compromise E3UBQ ligase activity, suppressing PTI.

ZtSSP2 has homologues in other members of the Dothideomycetes class, including the pine infecting hemibiotroph *D. septosporum*, banana infecting *P. fijiensis*, and barley pathogen *R. collo-cygni*. This broad conservation suggests that ZtSSP2 may be a core effector candidate in dothideomycete pathogens. Of the homologous proteins identified thirteen of these are from plant pathogens (Fig. 2b). We explored a possible interaction between *R. collo-cygni,* RcSSP2 and barley HvE3UBQ. However, we did not observe a strong interaction of RcSSP2 (homologue of ZtSSP2 with 51.67% similarity) with HvE3UBQ or $HvE3UBQ_{126\text{-}219}$ however, ZtSSP2 was found to interact with $HvE3UBQ_{126\text{-}219}$ (Fig. S4) demonstrating this plant host target is conserved. Homologues do also exist in two eurotiomycetes (*Cyphellophora europaea* and *Fonsecaea monophora*, 40-45% homology) which can cause disease in humans (de Hoog *et al.,* 2000; Queiróz *et al.,* 2018). In the outgroup of other dothidoemycetes which are non-pathogenic (Fig. 2b) some of these have been reported to be lichen forming fungi. Recently lichen forming fungi were reported to express SSPs (Armaleo *et al.,* 2019).

A range of virulence effectors from plant pathogens concentrate into a limited number of host cellular targets “Hubs” to subvert the host defence and enhance virulence (Mukhtar *et al*., 2011). One of the key regulatory networks in plant defence is the ubiquitin-proteasome system (UPS). This UPS regulates multiple aspects of plant immunity involving recognition, receptor protein accumulation and subsequent defence signalling (Marino *et al*., 2012; Ustun *et al*., 2016). Therefore, manipulation of host UPS by effectors is central to increasing pathogen virulence. Here, the *Z. tritici* candidate effector *ZtSSP2* was found to physically interact with a wheat host E3 Ubiquitin ligase (*TaE3UBQ*) both in yeast and *in planta*. Domain analysis of *TaE3UBQ* showed that it possesses a conserved RING-finger domain and four transmembrane domains accompanied by an extracellular loop in the middle (Fig.

6a, S5). The localisation of *TaE3UBQ* in *N. benthamiana* leaves suggests that TaE3UBQ could be localised predominantly to the cell periphery as a membrane localised protein. RING-finger domains are characteristic of RING-class E3 ubiquitin protein ligases that transfer ubiquitin from an E2 enzyme to a substrate protein. The RING domain mediates the interaction with the appropriate E2 enzyme (Van Wijk *et al.,* 2009). These E3 ligases are central to plant immune responses and are also known targets for pathogen secreted effector proteins. One such example was effector *AvrPiz-t* from the blast fungus *M. oryzae. AvrPiz-t* has been shown to interact and inhibit the rice RING-type E3 Ubiquitin ligase (APIP6) *in vitro* resulting in the suppression of the APIP6 mediated PTI response (Park *et al*., 2012). The effector AVR3a from *P. infestans* interacts and stabilizes the host U-box E3 ligase CMPG1 (Cys, Met, Pro and Gly protein 1) that is required for INF-1 triggered cell death. The Avr3a interaction with CMPG1 leads to CMPG1 modification and thus prevents host cell death induction during infection (Bos *et al*., 2010).

In wheat we found three homoalleles of E3UBQ gene on chromosome 1DS, 1A and 1B and we hypothesize that all of them could be targets of ZtSSP2 as they appear to be conserved (Fig. S6). For example, this was also observed for the stripe rust effector PEC6 that interacts with wheat adenosine kinases to suppress wheat defence (Liu *et al*., 2016). To understand the role of *TaE3UBQ* during *Z. tritici* infection, we silenced the gene transcript using BSMV VIGS. Silencing of *TaE3UBQ* resulted in increased *Z. tritici* symptoms. We speculate that ZtSSP2 binding to *TaE3UBQ* suppresses the wheat ubiquitin system and PTI. There exists accumulating evidence that E3 ubiquitin ligase is a central regulator of plant immunity and signalling (Trujillo and Shirasu, 2010; Marino *et al*., 2012). In rice, the resistance gene *Xa21* (*Xanthomonas oryzae pv. oryzae locus* 21) was shown to require a RING- E3 ubiquitin XA21 binding protein 3 (XB3) which plays a key role in accumulation of the XA21 protein and Xa21 mediated disease resistance (Wang *et al*., 2006). In *Arabidopsis,* the Plant U-Box 12 (PUB12); a U-box E3 ligase is involved in the PTI response against bacterial flagellin through Flagellin sensing 2 (FLS2) (Lu *et al*., 2011). Similarly, *Arabidopsis Tóxicos en Levadura* (ATL) family of RING finger E3 ligase (ATL9) is induced by fungal chitin and is involved in resistance against the biotrophic fungal pathogen, *Golovinomyces cichoracearum* (Deng *et al*., 2017).

In conclusion, the ZtSSP-TaE3UBQ interaction may modulate and compromise E3UBQ ligase activity suppressing wheat PTI. Virus-mediated gene silencing of TaE3UBQ was found to promote STB susceptibility which agrees with TaE3UBQ being a positive regulator of PTI. However, further work is needed to explore the outcome of ZtSSP2 interaction on TaE3UBQ activity and to identify downstream host interactors of TaE3UBQ which will provide information on how TaE3UBQ might regulate immunity in wheat.

Supporting Information

Fig. S1 *In silico* selection of non-annotated small secreted proteins (ZtSSP) of *Z. tritici*. (a) Selection of small proteins, 492 proteins encoded by the *Z. tritici* genome with EST support (do Amaral et al., 2012). (b) Proteins were classified based on their size and proteins with 50-315 amino acid length were selected. (c) Numbers of cysteine residues in the selected mature proteins after removing signal peptide. (d) Proteins were annotated based on the presence of conserved functional domain.

Fig. S2 BSMV mediated gene silencing (VIGS) of *phytoene desaturase* (*PDS*) gene in wheat. Image of bleached PDS silenced fourth leaf at 10-14 days post inoculation with BSMV.

Fig. S3 Three homologues of *TaE3UBQ* are of high similarity. a) Nucleotide sequence alignments and (b) Amino acid sequences of three homologues of *TaE3UBQ* gene were obtained from Ensembl plant database. The three homologues are located on chromosomes 1A, 1B and 1D, respectively, base variations indicated with blue. The sequence alignment was performed using *MultAlin* (Corpet, 1998). Regions targeted by VIGS1 and VIGS2 constructs are highlighted below by a solid blue and green line respectively. Region targeted by conserved qPCR primers are shown by black arrows.

Fig. S4 Yeast two hybrid assay to test the interaction of RcSSP2 with HvE3UBQ. Yeast transformed with HvE3UBQ, $HvE3UBQ_{126\text{-}219}$ and RcSSP2 cloned in the Gal4 bait and prey vectors. Yeast were grown for 4-5 days under selective Trp/Leu/His drop out medium in presence of 0.3mM 3-amino-1,2,4-triazole (3-AT) and X-α-gal medium or a non-selective Trp/Leu drop out medium (-TL) conditions. BD represents Binding domain & AD, activating domain. Experiment was repeated three times independently with three replicates per independent experiment.

Fig. S5 TaE3UBQ protein topology illustration using MemBrain 3.1 (Yin *et al*., 2017). The protein sequence of TaE3UBQ shows transmembrane helix domains, an extracellular loop (155-218) and intracellular regions within the protein structure. The C3H2C3-ring is shown.

Fig. S6 Expression profiles of wheat E3 ubiquitin ligase homeologs during *Z. tritici* infection. Normalised expression data (in transcript per million (TPM)) retrieved from expVIP (Borrill *et al*., 2016) for all three TaE3UBQ homeologs following *Z. tritici* infection and mock treatment at 1, 4, 9, 14 and 21 days post inoculation (dpi).

Table S1. List of putative candidate effector proteins of *Z. tritici* (*ZtSSP*s). Gene ID corresponds to JGI gene ID, Gene annotations were obtained from JGI database and blast search performed with NCBI BlastP. Functional annotation was performed using NCBI CDD database. EffectorP 1.0 and ApoplasticP 1.0 was used to predict if small, secreted proteins are predicted effectors and their localisation respectively: '+' and '-' indicates positive and negative prediction. Candidates that matched Kettles *et al*., 2017 are referred as Zt.

Table S2. *Z. tritici* small, secreted proteins have potential homologues in other plant pathogens. 46 out of 90 ZtSSP's show homologues in other plant pathogens. JGI code, description, accession numbers, percentage identity and e-value. Cut-off of 50% percentage similarity and e-value 0.01 using NCBI Blast.

Table S3. Expression of seventeen conserved effector candidates across three *Z. tritici* isolates IPO323, 553.11 and 560.11 at 7 dpi. The Logarithmic Fold Changes is shown for each gene (LogFC), False Discovery Rate (FDR), FKPM values Fragments Per Kilobase Million (FKM) and Filtered Reads (genes with a total read count of <1 were filtered out of the analysis).

Table S4. List of primers used in this study.

Table S5. ZtSSP2 homologues in the Dothideomycetes with species, accession numbers, description, e-value, sequence, and length of the 20 closest homologues using NCBI BlastP. Red indicates signal peptide.

Table S6. List of wheat proteins identified as a potential interactors with *Z. tritici* candidate ZtSSP2. Sequences obtained from positive clones in yeast 2 hybrid assay was blasted against Ensemble wheat (TGACv1) dataset and corresponding full-length sequences were obtained.

Table S7. *Z. tritici* small, secreted proteins with potential homologues only in present in *Zymoseptoria brevis*. JGI code, description, accession numbers, percentage identity and e-value. Cut-off of 50% percentage similarity and e-value 0.01 using NCBI Blast.

Data availability

All data supporting the findings of this study are available within the paper and within its supplementary materials published online.

Acknowledgements

This work was funded by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 674964 (CerealPath), SFI Career Development Award (15/CDA/3451), SFI Strategic Partnerships Programme (16/SPP/3296), Irish Department of Agriculture, Food and the Marine Research (DAFM) Stimulus Project VICCI (14/S/819) and Science Foundation Ireland PI project 14/1A/2508. We would like to thank Dr. Eleanor Gilroy (James Hutton Institute) for providing us Inf-1 and GV3101 and Dr. Stephen Kildea (Teagasc, Carlow) for providing the 553.11 and 560.11 isolate.

Author contributions

S.J.K, F.D, P.S and A.F designed the experiments. S.J.K., A.R., D.D., M.M., B.Z. performed the experiments. S.J.K., A.R., analysed the data. S.J.K and A.F wrote the manuscript with input from D.D., P.S., J. B, F.D.

REFERENCES

Anders S. Pyl PT, Huber W. 2015. HTSeq: A Python framework to work with high-throughput sequencing data, *Bioinformatics*, **31**(2), 166-169

Andrews, S. 2010. FastQC: a quality control tool for high throughput sequence data.

Armaleo D, Müller O, Lutzon, F, Andrésson ÓS, Blanc G, Bode HB, ... & BB Xavier. 2019. The lichen symbiosis re-viewed through the genomes of *Cladonia grayi* and its algal partner *Asterochloris glomerata*. *BMC genomics*, **20**(1), 605.

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, **30**(15): 2114-2120.

Borrill, P, Ramirez-Gonzalez, R. and Uauy, C. 2016. expVIP: a customizable RNA-seq data analysis and visualization platform. *Plant physiology*, **170** (4): 2172-2186.

Bos JIB, Armstrong MR, Gilroy EM, Boevink PC, Hein I, Taylor RM, Zhendong T, Engelhardt S, Vetukuri RR, Harrower B. 2010. *Phytophthora infestans* effector AVR3a is essential for virulence and manipulates plant immunity by stabilizing host E3 ligase CMPG1. *Proceedings of the National Academy of Sciences*, USA **107,** 9909–9914.

Brading, PA, Verstappen EC, Kema GH, Brown JK. 2002. A gene-for-gene relationship between wheat and Mycosphaerella graminicola, the Septoria tritici blotch pathogen. *Phytopathology*, **92**(4): 439-445.

Brady NC. 1983. *Advances in agronomy* (Vol. 36). Academic Press.

Brennan, CJ, Zhou B, Benbow HR, Ajaz S, Karki SJ, Hehir JG, O'driscoll A, Feechan A, Mullins E. and Doohan FM. 2020. Taxonomically restricted wheat genes interact with small secreted fungal proteins and enhance resistance to Septoria tritici blotch disease. *Frontiers in Plant Science*, 11, p.433.

Chen S, Songkumarn P, Venu RC, Gowda M, Bellizzi M, Hu J, Liu W, Ebbole D, Meyers B, Mitchell T, Wang GL. 2013. Identification and characterization of in planta–expressed secreted effector proteins from *Magnaporthe oryzae* that induce cell death in rice. *Molecular plant-microbe interactions*, *26*(2): 191-202.

Corpet, F. 1988. Multiple sequence alignment with hierarchical clustering. *Nucleic acids research*, *16*(22), pp.10881-10890.

de Hoog G.S, Mayser P, Haase G, Horré R, Horrevorts, A.M. 2000. A new species, *Phialophora europaea*, causing superficial infections in humans. *Mycoses*, **43**(11-12), p.409.

Deng F, Guo T, Lefebvre M, Scaglione S, Antico CJ, Jing T, Yang X, Shan W, Ramonell KM. 2017. Expression and regulation of ATL9, an E3 ubiquitin ligase involved in plant defense. *PloS one*, **12**(11), p.e0188458.

Deslandes L, Rivas S. 2012. Catch me if you can: bacterial effectors and plant targets. *Trends in plant science*. **17**(11): 644-655.

do Amaral AM, Antoniw J, Rudd JJ and Hammond-Kosack KE. 2012. Defining the predicted protein secretome of the fungal wheat leaf pathogen *Mycosphaerella graminicola*. *PLoS One*, **7**(12), p.e49904.

Dou D, Zhou JM. 2012. Phytopathogen effectors subverting host immunity: different foes, similar battleground. *Cell host & microbe*. **12**(4): 484-495.

Douchkov D, Nowara D, Zierold U, Schweizer P. 2005. A high-throughput gene-silencing system for the functional assessment of defense-related genes in barley epidermal cells. *Molecular Plant-Microbe Interactions*, **18**(8), 755-761.

Earley KW, Haag JR, Pontes O, Opper K, Juehne T, Song K, Pikaard CS. 2006. Gateway-compatible vectors for plant functional genomics and proteomics. *The Plant Journal*, **45**(4), 616-629.

Eyal Z, Scharen AL, Huffman MD, Prescott JM. 1985. Global insights into virulence frequencies of Mycosphaerella graminicola. *Phytopathology*, **75**(12),1456-1462.

Fernandez-Pozo N, Rosli HG, Martin GB, Mueller LA. 2015. The SGN VIGS tool: user-friendly software to design virus-induced gene silencing (VIGS) constructs for functional genomics. *Molecular plant*, **8**(3), 486-488.

Fones H, Gurr S. 2015. The impact of Septoria tritici Blotch disease on wheat: An EU perspective. *Fungal Genetics and Biology*, **79**,3-7.

Fones, H.N., Eyles, C.J., Kay, W., Cowper, J. and Gurr, S.J., 2017. A role for random, humidity-dependent epiphytic growth prior to invasion of wheat by Zymoseptoria tritici. *Fungal Genetics and Biology*, **106**, pp.51-60.

Franco-Orozco B, Berepiki A, Ruiz O, Gamble L, Griffe LL, Wang S, Birch PR, Kanyuka K, Avrova A. 2017. A new proteinaceous pathogen-associated molecular pattern (PAMP) identified in Ascomycete fungi induces cell death in Solanaceae. *New Phytologist*, **214**(4), 1657-1672.

Gehl C, Waadt R, Kudla J, Mendel RR, Hänsch R. 2009. New GATEWAY vectors for high throughput analyses of protein–protein interactions by bimolecular fluorescence complementation. *Molecular plant*, **2**(5), 1051-1058.

Goodwin, S.B., M'barek, S.B., Dhillon, B., Wittenberg, A.H., Crane, C.F., Hane, J.K. Foster, A.J., Van der Lee, T.A., Grimwood, J., Aerts, A. and Antoniw, J. 2011. Finished genome of the fungal wheat pathogen *Mycosphaerella graminicola* reveals dispensome structure, chromosome plasticity, and stealth pathogenesis. *PLoS Genet*, **7**(6), p.e1002070.

Hogenhout SA, Van der Hoorn RA, Terauchi R, Kamoun S. 2009. Emerging concepts in effector biology of plant-associated organisms. *Molecular plant-microbe interactions*, **22**(2),115-122.

Holzberg S, Brosio P, Gross C, Pogue GP. 2002. Barley stripe mosaic virus-induced gene silencing in a monocot plant. *The Plant Journal*, **30**(3), 315-327.

Jones JD, Dangl JL. 2006. The plant immune system. *Nature*, **444**(7117): 323-329.

Kema GH, Gohari AM, Aouini L, Gibriel HA, Ware SB, van Den Bosch F, Manning-Smith R, Alonso-Chavez V, Helps J, M'Barek SB, Mehrabi R. 2018. Stress and sexual reproduction affect the dynamics of the wheat pathogen effector AvrStb6 and strobilurin resistance. *Nature genetics*, **50**(3), 375.

Kema GH, van Silfhout CH. 1997. Genetic variation for virulence and resistance in the wheat-Mycosphaerella graminicola pathosystem III. Comparative seedling and adult plant experiments. *Phytopathology*, **87**(3), 266-272.

Kettles GJ, Bayon C, Canning G, Rudd JJ, Kanyuka K. 2017. Apoplastic recognition of multiple candidate effectors from the wheat pathogen *Zymoseptoria tritici* in the nonhost plant *Nicotiana benthamiana*. *New Phytologist*, **213**(1), 338-350.

Kettles GJ, Bayon C, Sparks CA, Canning G, Kanyuka K, Rudd JJ. 2018. Characterization of an antimicrobial and phytotoxic ribonuclease secreted by the fungal wheat pathogen *Zymoseptoria tritici*. *New Phytologist*, **217**(1), 320-331.

Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R. Salzberg SL. 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biology*, **14**, R36.

Kumar S, Stecher G, Tamura, K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular biology and evolution*, **33**(7), 1870-1874.

Lee WS, Rudd JJ, Hammond-Kosack KE, Kanyuka K. 2014. *Mycosphaerella graminicola* LysM effector-mediated stealth pathogenesis subverts recognition through both CERK1 and CEBiP homologues in wheat. *Molecular Plant-Microbe Interactions*, **27**(3), 236-243.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R. and 1000 Genome Project Data Processing Subgroup. 2009. The Sequence alignment/map (SAM) format and SAMtools. *Bioinformatics*, **25**, 2078-2079.

Liu C, Pedersen C, Schultz-Larsen T, Aguilar GB, Madriz-Ordeñana K, Hovmøller MS,Thordal-Christensen H. 2016. The stripe rust fungal effector PEC 6 suppresses pattern-triggered immunity in a host species-independent manner and interacts with adenosine kinases. *New Phytologist*.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *methods*, **25**(4), 402-408.

Lu D, Lin W, Gao X, Wu S, Cheng C, Avila J, Heese A, Devarenne TP, He P, Shan L. 2011. Direct ubiquitination of pattern recognition receptor FLS2 attenuates plant innate immunity. *Science*, **332**(6036), 1439-1442.

Lynch KM, Zannini E, Guo J, Axel C, Arendt EK, Kildea S, Coffey A. 2016. Control of *Zymoseptoria tritici* cause of septoria tritici blotch of wheat using antifungal Lactobacillus strains. *Journal of applied microbiology*, **121**(2), 485-494.

M'Barek SB, Cordewener JH, Ghaffary SMT, van der Lee TA, Liu Z, Gohari AM, Mehrabi R, America AH, Robert O, Friesen TL, Hamza S. 2015. FPLC and liquid-chromatography mass spectrometry identify candidate necrosis-inducing proteins from culture filtrates of the fungal wheat pathogen *Zymoseptoria tritici*. *Fungal Genetics and Biology*, **79**, 54-62.

Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, Fong JH, Geer LY, Geer RC, Gonzales NR, Gwadz M. 2010. CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic acids research*, **39(**suppl_1), D225-D229.

Marino D, Peeters N, Rivas S. 2012. Ubiquitination during plant immune signaling. Plant physiology, **160**(1): 15-27.

Marshall R, Kombrink A, Motteram J, Loza-Reyes E, Lucas J, Hammond-Kosack KE, Thomma BP, Rudd JJ. 2011. Analysis of two in planta expressed LysM effector homologues from the fungus *Mycosphaerella graminicola* reveals novel functional properties and varying contributions to virulence on wheat. *Plant physiology*, **156**(2), 756-769.

McLellan, H., Chen, K., He, Q., Wu, X., Boevink, P.C., Tian, Z. and Birch, P.R. 2020. The ubiquitin E3 ligase PUB17 positively regulates immunity by targeting a negative regulator, KH17, for degradation. *Plant Communications*, p.100020.

Meile L, Croll D, Brunner PC, Plissonneau C, Hartmann FE, McDonald BA, Sánchez-Vallet A. 2018. A fungal avirulence factor encoded in a highly plastic genomic region triggers partial resistance to septoria tritici blotch. *New Phytologist*, **219**(3), 1048-1061.

Mirzadi Gohari A, Ware SB, Wittenberg AH, Mehrabi R, Ben M'Barek S, Verstappen EC, van der Lee TA, Robert O, Schouten H.J. de Wit PP, Kema GH. 2015. Effector discovery in the fungal wheat pathogen *Zymoseptoria tritici*. *Molecular plant pathology*, **16**(9), 931-945.

Motteram J, Küfner I, Deller S, Brunner F, Hammond-Kosack KE, Nürnberger T, Rudd JJ. 2009. Molecular characterization and functional analysis of MgNLP, the sole NPP1 domain–containing protein, from the fungal wheat leaf pathogen *Mycosphaerella graminicola*. *Molecular Plant-Microbe Interactions*, **22**(7), 790-799.

Mukhtar MS, Carvunis AR, Dreze M, Epple P, Steinbrenner J, Moore J, Tasan M, Galli M, Hao T, Nishimura MT, Pevzner SJ. 2011. Independently evolved virulence effectors converge onto hubs in a plant immune system network. *science*, **333**(6042): 596-601.

NakagawaT, Kurose T, Hino T, Tanaka K, Kawamukai M, Niwa Y, Toyooka K, Matsuoka K, Jinbo T, Kimura T. 2007. Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *Journal of bioscience and bioengineering*, **104**(1), 34-41.

Nelson BK, Cai X, Nebenführ A. 2007. A multicolored set of in vivo organelle markers for co-localization studies in Arabidopsis and other plants. *The Plant Journal*, **51**(6), 1126-1136.

Nielsen, H. 2017. Predicting secretory proteins with SignalP. In *Protein function prediction* pp. 59-73. Humana Press, New York, NY.

Park CH, Chen S, Shirsekar G, Zhou B, Khang CH, Songkumarn P, Afzal AJ, Ning Y, Wang R, Bellizzi M, Valent B. 2012. The *Magnaporthe oryzae* effector AvrPiz-t targets the RING E3 Ubiquitin Ligase APIP6 to suppress pathogen-associated molecular pattern–triggered immunity in rice. *The Plant Cell*, **24** (11): 4748-4762.

Park, C.H., Shirsekar, G., Bellizzi, M., Chen, S., Songkumarn, P., Xie, X., Shi, X., Ning, Y., Zhou, B., Suttiviriya, P. and Wang, M. 2016. The E3 ligase APIP10 connects the effector AvrPiz-t to the NLR receptor Piz-t in rice. *PLoS pathogens*, **12** (3), p.e1005529.

Perochon A, Dieterle S, Pouzet C, Aldon D, Galaud JP, Ranty B. 2010. Interaction of a plant pseudo-response regulator with a calmodulin-like protein. *Biochemical and biophysical research communications*, **398**(4), 747-751.

Pliego C, Nowara D, Bonciani G, Gheorghe DM, Xu R, Surana P, Whigham E, Nettleton D, Bogdanove AJ, Wise RP, Schweizer P. 2013. Host-induced gene silencing in barley powdery mildew reveals a class of ribonuclease-like effectors. *Molecular Plant-Microbe Interactions*, **26**(6), 633-642.

Queiróz A.J, Pereira Domingos F, Antônio J.R, 2018. Chromoblastomycosis: clinical experience and review of literature. International journal of dermatology, **57**(11), pp.1351-1355.

R Core Team. 2016. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for statistical Computing. https://www.R-project.org/

Rafiqi M, Jelonek L, Akum NF, Zhang F, Kogel KH. 2013. Effector candidates in the secretome of *Piriformospora indica*, a ubiquitous plant-associated fungus. *Frontiers in plant science*, **4**,228.

Robinson MD, Oshlack A. 2010. A scaling normalization method for di erential expression analysis of RNA-seq data. *Genome Biology, **11***, R25.

Robinson MD, Smyth GK. 2008. Small sample estimation of negative binomial dispersion, with applications to SAGE data. *Biostatistics*, **9**, 321-332.

Rudd JJ, Kanyuka K, Hassani-Pak K, Derbyshire M, Andongabo A, Devonshire J, Lysenko A, Saqi M, Desai NM, Powers SJ, Hooper J. 2015. Transcriptome and metabolite profiling of the infection cycle of *Zymoseptoria tritici* on wheat reveals a biphasic interaction with plant immunity involving differential pathogen chromosomal contributions and a variation on the hemibiotrophic lifestyle definition. *Plant physiology*, **167**(3), 1158-1185.

Sánchez-Vallet, A., McDonald, M.C., Solomon, P.S. and McDonald, B.A. 2015. Is *Zymoseptoria tritici* a hemibiotroph? *Fungal Genetics and Biology*, **79,** 29-32.

Schweizer P, Pokorny J, Schulze-Lefert P, Dudler R. 2000. Double-stranded RNA interferes with gene function at the single-cell level in cereals. *The Plant Journal*, **24**(6), 895-903.

Scofield SR, Huang L, Brandt AS, Gill BS. 2005. Development of a virus-induced gene-silencing system for hexaploid wheat and its use in functional analysis of the Lr21-mediated leaf rust resistance pathway. *Plant Physiology*, **138**(4), 2165-2173.

Shetty, N.P., Kristensen, B.K., Newman, M.A., Møller, K., Gregersen, P.L. and Jørgensen, H.L. 2003. Association of hydrogen peroxide with restriction of Septoria tritici in resistant wheat. *Physiological and Molecular Plant Pathology*, **62**(6), pp.333-346.

Sperschneider J, Dodds PN, Singh KB,Taylor JM. 2018. ApoplastP: prediction of effectors and plant proteins in the apoplast using machine learning. *New Phytologist*, **217**(4), 1764-1778.

Sperschneider J, Gardiner DM, Dodds PN, Tini F, Covarelli L, Singh KB, Manners JM, Taylor JM. 2016. EffectorP: predicting fungal effector proteins from secretomes using machine learning. *New Phytologist*, **210**(2), 743-761.

Stergiopoulos I, de Wit PJ. 2009. Fungal effector proteins. *Annual review of phytopathology*. **47:** 233-263.

Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L. 2010. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Natural Biotechnology*, **28**, 511-515.

Trujillo M, Shirasu K. 2010. Ubiquitination in plant immunity. *Current Opinion in Plant Biology* **13**: 402–408

Üstün S, Sheikh A, Gimenez-Ibanez S, Jones AM, Ntoukakis V, Börnke F. 2016. The proteasome acts as a hub for plant immunity and is targeted by Pseudomonas type-III effectors. *Plant physiology*, pp.-00808.

Van Wijk SJ, De Vries SJ, Kemmeren P, Huang A, Boelens R, Bonvin AM, Timmers HTM. 2009. A comprehensive framework of E2–RING E3 interactions of the human ubiquitin–proteasome system. *Molecular systems biology*, **5**(1).

Váry Z, Mullins E, McElwain JC, Doohan, FM. 2015. The severity of wheat diseases increases when plants and pathogens are acclimatized to elevated carbon dioxide. *Global change biology*, **21**(7), 2661-2669.

Wang YS, Pi LY, Chen X, Chakrabarty PK, Jiang J, De Leon AL, Liu GZ, Li L, Benny U, Oard J, Ronald PC. 2006. Rice XA21 binding protein 3 is an ubiquitin ligase required for full Xa21-mediated disease resistance. *The Plant Cell*, **18**(12):3635-3646.

Yin X, Yang J, Xiao F, Yang Y, Shen, H.B. 2018. MemBrain: an easy-to-use online webserver for transmembrane protein structure prediction. *Nano-micro letters*, **10**(1), 2.

Zhong Z, Marcel TC, Hartmann FE, Ma X, Plissonneau C, Zala M, Ducasse A, Confais J, Compain J, Lapalu N, Amselem J. 2017. A small secreted protein in *Zymoseptoria tritici* is responsible for avirulence on wheat cultivars carrying the Stb6 resistance gene. *New Phytologist*, **214**(2), 619-631.

Zhou B, Benbow HR, Brennan CJ, Arunachalam C, Karki SJ., Mullins E, Feechan A, Burke JI, Doohan FM. 2020. Wheat Encodes Small, Secreted Proteins That Contribute to Resistance to Septoria Tritici Blotch. *Frontiers in Genetics*, **11**, 469.

Figure Legends

Fig. 1. ZtSSP2 is a secreted protein. (a) Design of the gateway compatible yeast pGADT7-ZtSSP2-Suc2 vector and Invertase mutant yeast strain SEY6210 used for the secretion assay (Brennan *et al.,* 2020). (b) Yeast strain carrying *ZtSSP2* with secretion signal fused in frame with the Invertase gene Suc2 were able to grow in sucrose containing drop out media (SD-TL) sucrose therefore cells will grow if Invertase is secreted. SEY6210 carrying pGAD-ΔSP:SUC2$^{22\text{-}5112}$ vector was used as negative control while SEY6210 with pGAD-SUC2$^{\text{Full length}}$ acts as positive control. This experiment was repeated three times independently with three replicates per independent experiment.

Fig. 2. ZtSSP2 (*Z. tritici* small, secreted protein candidate 2) homologues are widely present across Dothideomycetes. (a) Protein alignment using ClustalW, of the full protein sequence of *Z. tritici* ZtSSP2 with homologues from seven plant infecting Dothideomycetes fungi. Asterisk indicates conserved cysteine residues and shading represents identical or similar amino acids. Signal peptide predicted using SignalP 4.1 (indicated by red line) (b) The Unrooted maximum likelihood phylogeny of ZtSSP2 and the 20 closest orthologues including plant pathogens (green), other non-pathogenic Dothideomycetes (orange) and Eurotiomycetes (blue) which are human pathogens. Tree was generated with MEGA7 (Kumar *et al*. 2016), Bootstrap values are based on 1000 replications. Sequences were obtained by blastp (NCBI) and aligned using ClustalW.

Fig. 3. *ZtSSP2* is expressed at different stages of *Z. tritici* infection (2-10 dpi biotrophic and 14-21 dpi necrotrophic stage). Gene expression analysis of *ZtSSP2* un-infected control, at 2, 4, 8, 10, 14, 21 dpi of wheat (cv.Remus) with *Z. tritici* isolate 560.11 (Lynch *et al*. 2016). Control plants were inoculated with Tween 20. RNA was extracted from three wheat leaves per time point followed by reverse transcription into cDNA. The qPCR was performed on cDNA using specific primers for *ZtSSP2*. The expression levels of *β-tubulin* (*Z. tritici*) were used to normalize the expression levels of *ZtSSP2*. Each independent experiment had three leaves each from three individual plants. The bars represent the mean relative expression ± SEM of three independently replicated experiments. Different letters above bars indicate significant differences, as determined by Tukey's test (*P<0.05).

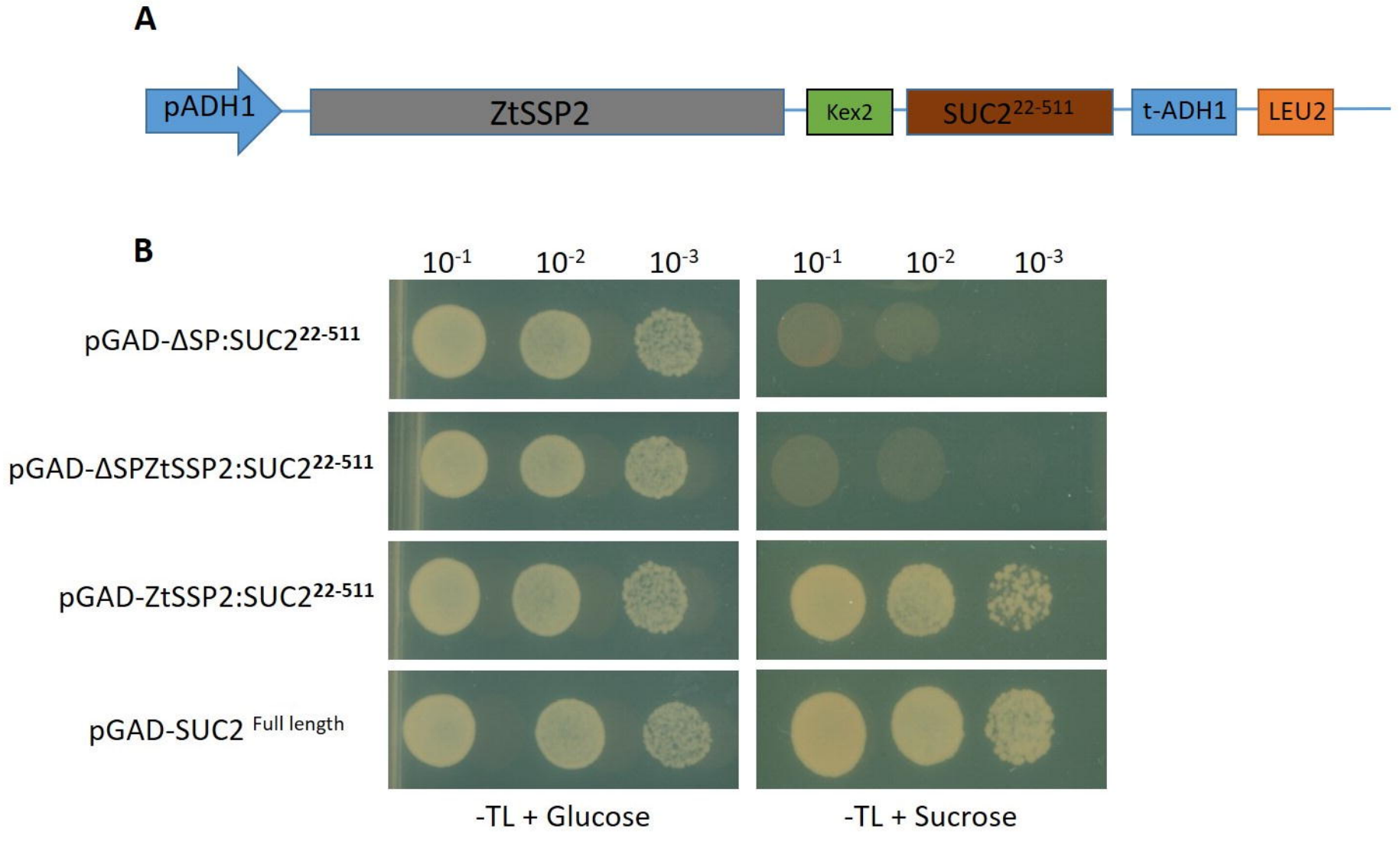
Fig. 4 Single cell death assay in wheat leaves. Wheat leaves cv. Kanzler were co-bombarded with pUbiGus as a transformation marker, pEG101 for overexpression of *ZtSSP2* (PEG101:ZtSSP2), and the *B-Peru/C1-expression* plasmid pBC17 that induces anthocyanin accumulation in wheat epidermal cells. **(a)** Unstained wheat leaf showing epidermal cells that accumulate anthocyanin (arrow; bar=20 µm) 4 days after bombardment and **(b)** β-glucuronidase (GUS) expressing cells after GUS staining. **(c)** Quantification of the relative number of anthocyanin producing cells calculated as the ratio of anthocyanin accumulated cells to the number of GUS expressing cells. Co-transformation of pBC17 and pUbiGUS with the empty vector pEG101 and pEG101: *Zt-6* served as a positive control inducing cell death. Values

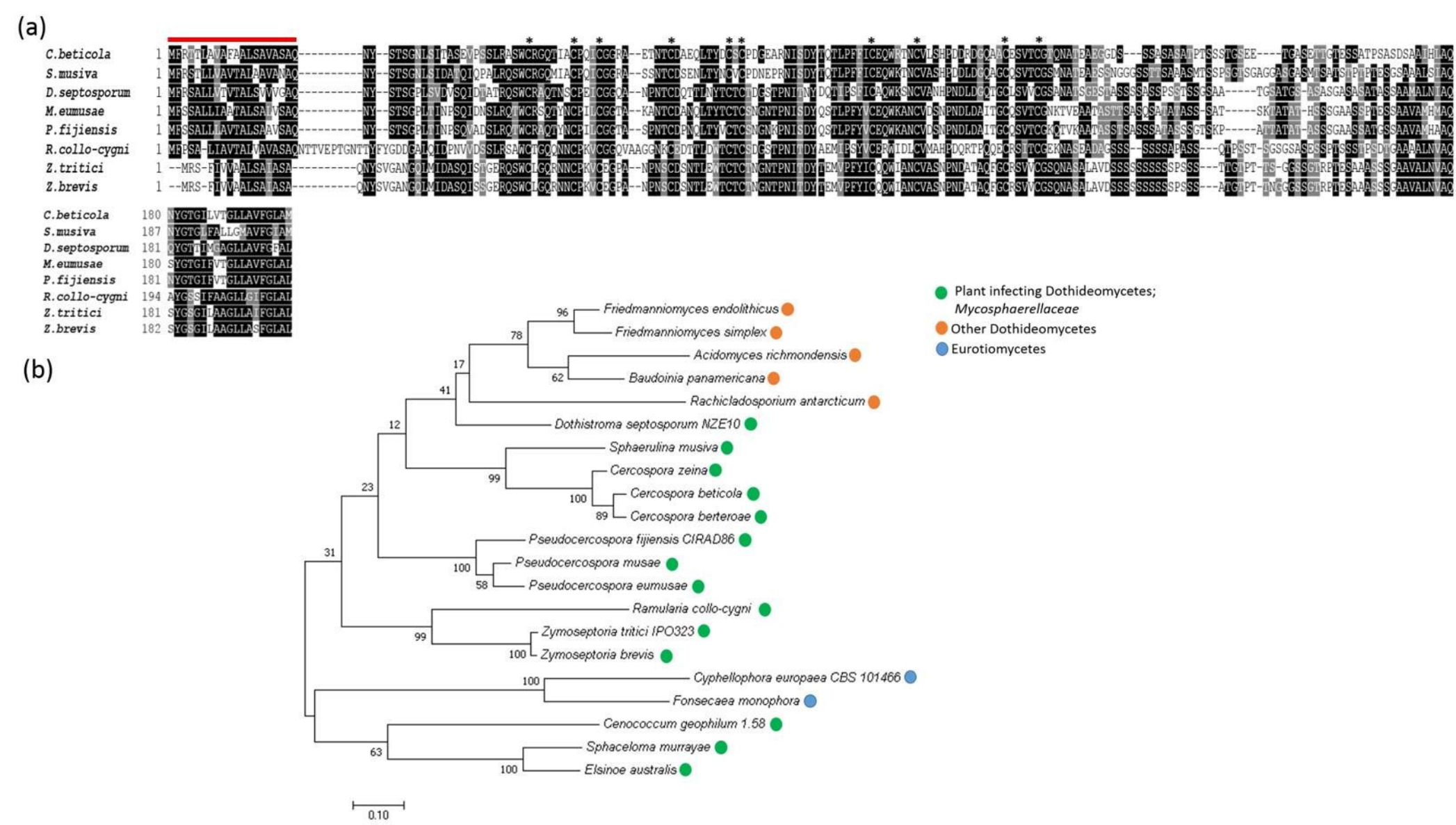
are the means of four independent experiments with 7 leaves counted per repetition (Bars ±SEM). The asterisk on top of bar represents significant differences determined by Tukey test (* for $P<0.05$).

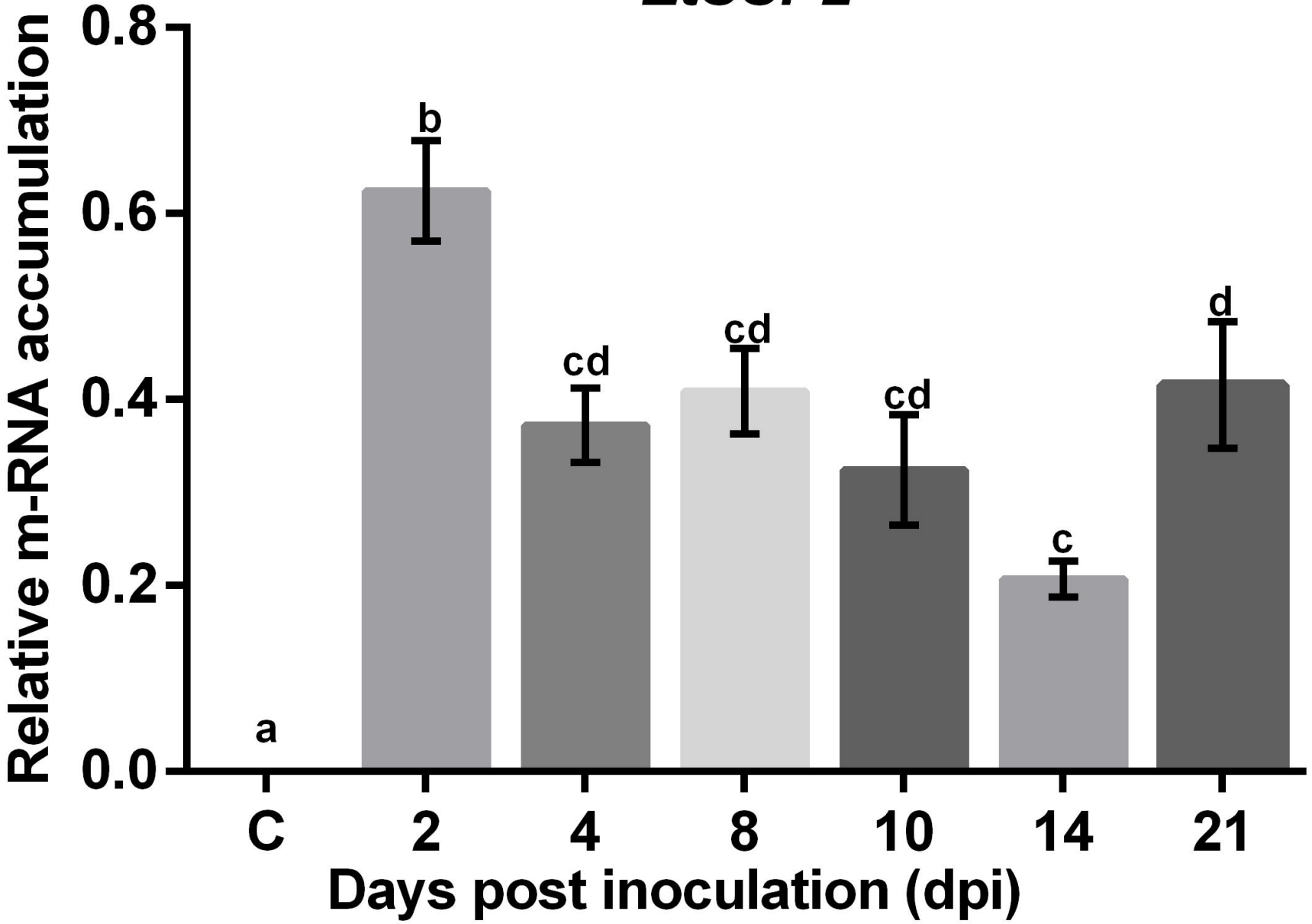
Fig. 5 Interaction of (ΔSP) *ZtSSP2* with host protein*TaE3UBQ*. **(a)** Yeast two-hybrid assay using the yeast cells transformed with *TaE3UBQ* and (ΔSP) *ZtSSP2* cloned in the Gal4 bait (BD) and prey vectors (AD). Yeast strain Y2HGold co-expressing the vector BD containing *TaE3UBQ*, $TaE3UBQ_{126\text{-}219}$, TaSSP6 or empty bait vector (BD-X) and the prey vector containing *ZtSSP2*, *Zt10, Zt06* or empty vector (AD-X) were grown on auxotrophic media (SD/-Leu-Trp) (left panel) or selective Trp/Leu/His drop out medium in presence of 0.5 mM 3-amino-1,2,4-triazole (3-AT). Only yeast cells co-expressing *ZtSSP2* and $TaE3UBQ_{126\text{-}219}$ grew on selective media (SD/-Leu-Trp-His) (middle panel) and showed α-galactosidase activity encoded by α-galactosidase (MEL1) (right panel). BD-TaSSP6 and AD-Zt06 was used as positive control (Zhou *et al.,* 2020). The experiment was repeated independently three times, three plates per experiment with similar results. (b) Validation of *in planta* interaction of (ΔSP) *ZtSSP2 with* wheat Ubiquitin protein visualized by the bimolecular fluorescence complementation (BiFC) assay. Confocal microscopy images of representative *N. benthamiana* epidermal leaf cells expressing proteins fused to the N- or C-terminal part of the YFP as indicated. YFP, brightfield are shown both separately and as an overlay. Scale bar is 10 µm. In both experiment a and b, candidate Zt-10 was used as a negative control while Cnx6 homodimerisation was used as BiFC positive control. Experiments were repeated at least two times independently with similar results (three leaves each from individual plants). (c) Confirmation of the interaction between ZtSSP2 and TaE3UBQ by co-immunoprecipitation assays. Western blot of total proteins from *N. benthamiana* leaves co-infiltrated with the construct and co-immunoprecipitated using GFP-Trap magnetic beads. Expression of constructs in the leaves is indicated by '+'. Immunoblots were performed using anti-GFP and anti-HA antibodies. The protein size markers are indicated in kDa.

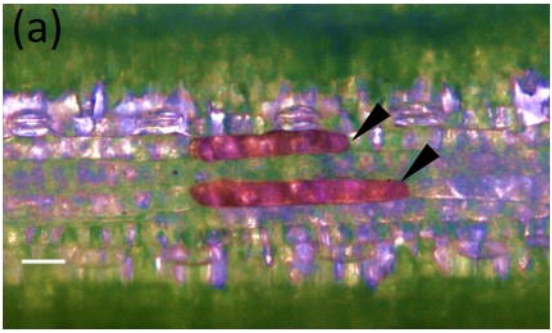
Fig. 6 Sequence analysis of wheat E3 ubiquitin ligase, *T. aestivum* E3 UBQ ligase protein (*TaE3UBQ*). **(a)** Schematic representation of the structure of *TaE3UBQ* and the conserved RING finger motif using TMpred and CCD domain database. (b) Sequence alignment of the C3H2C3-type RING finger conserved motif in TaE3UBQ homologues in Barley (Gene Bank accession no. BAJ95361.1), Brachypodium (XP_003568812.1), Oryzae (XP_015640584.1), Maize (PWZ17186.1), Arabidopsis (NP_178156.1), Millet (RLM97696.1), Sorghum (XP_002439378.1). Putative Zn^{2+}-interacting amino acid residues are indicated by red while, conserved and non-conserved residues are highlighted by black and white, respectively. (c) GFP-TaE3UBQ fluorescent signal localises to the cell periphery similar to the signal obtained from a plasma membrane marker pm-yk (Nelson *et al*., 2007) in *N. benthamiana* cells. The GFP fluorescent protein was found distributed throughout the cell of transformed *N. benthamiana* leaf cells. Bars = 20 µm

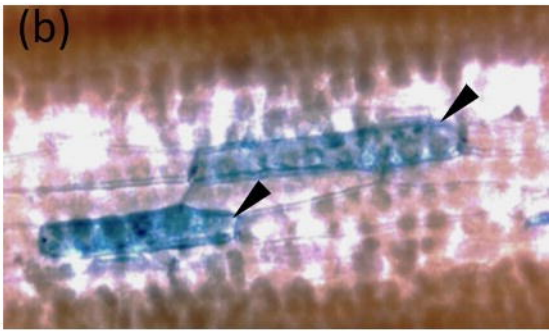
Fig. 7 BSMV mediated virus induced gene silencing (VIGS) of wheat E3 ubiquitin ligase (*TaE3UBQ*) resulted in increased *Z. tritici* susceptibility of wheat leaves. Wheat leaves at the second leaf stage (cv. Longbow) were treated with either FES (VIGS buffer), BSMV:00 (empty vector) or BSMV:UBQ_V1 or BSMV:UBQ_V2 (constructs targeting all three homeologs of TaE3UBQ). The third and fourth leaves of VIGS treated plants were inoculated with either mock (Tween) or *Z. tritici* (560.11). Subsequently, the third leaves were collected for gene expression studies and the fourth leaves for phenotyping and spore count. (a) Relative transcript abundance of all *TaE3UBQ* homeologs in knockdown plants (5-6 leaves each from individual seedlings per treatment per replicate). (b, c) Phenotype of VIGS treated leaves infected with *Z. tritici* at 21 days post inoculation (10 leaves each from individual seedlings per treatment per replicate). (d) Average spore counts per ml were counted over 2 cm leaf lengths (7 leaves each from individual seedlings per treatment per replicate). Three independent experiments were carried out. Bars with the same letter are not significantly different. ($P < 0.05$).


A
pADH1
ZtSSP2
Kex2
SUC2 22-511
t-ADH1
LEU2
B
10-1
10-2
10-3
10-1
10-2
10-3
pGAD-ΔSP:SUC2 22-511
pGAD-ΔSPZtSSP2:SUC2 22-511
pGAD-ZtSSP2:SUC2 22-511
pGAD-SUC2 Full length
-TL + Glucose
-TL + Sucrose

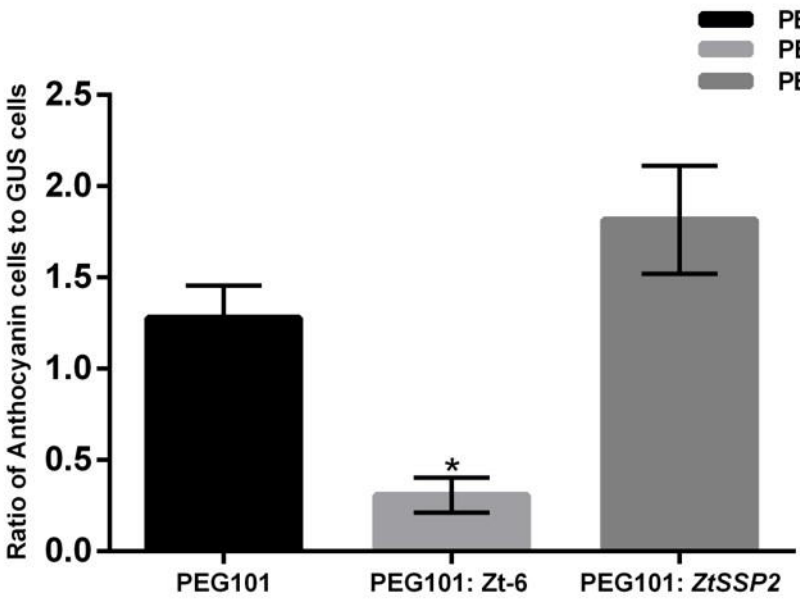

(a)
C.beticola
S.musiva
D.septosporum
M.eumusae
P.fijiensis
R.collo-cygni
Z.tritici
Z.brevis
(b)
Plant infecting Dothideomycetes; Mycosphaerellaceae
Other Dothideomycetes
Eurotiomycetes
Friedmanniomyces endolithicus
Friedmanniomyces simplex
Acidomyces richmondensis
Baudoinia panamericana
Rachicladosporium antarcticum
Dothistroma septosporum NZE10
Sphaerulina musiva
Cercospora zeina
Cercospora beticola
Cercospora berteroae
Pseudocercospora fijiensis CIRAD86
Pseudocercospora musae
Pseudocercospora eumusae
Ramularia collo-cygni
Zymoseptoria tritici IPO323
Zymoseptoria brevis
Cyphellophora europaea CBS 101466
Fonsecaea monophora
Cenococcum geophilum 1.58
Sphaceloma murrayae
Elsinoe australis
0.10

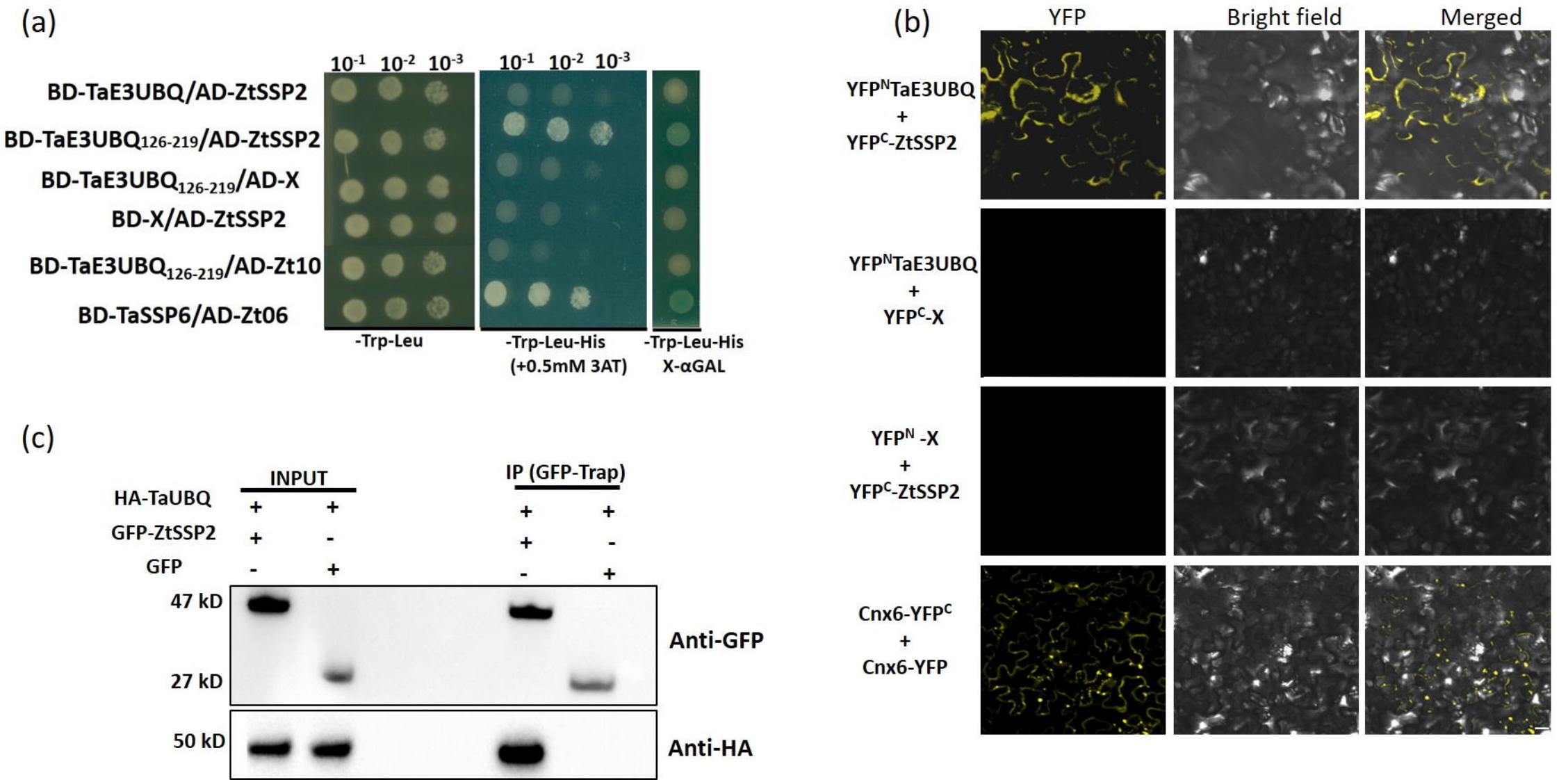

ZtSSP2
Relative m-RNA accumulation
0.8
0.6
0.4
0.2
0.0
a
b
cd
cd
cd
c
d
C
2
4
8
10
14
21
Days post inoculation (dpi)


(a)

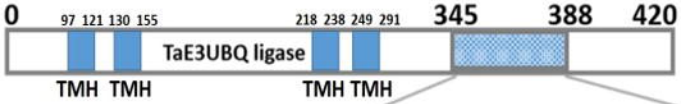

(b)

(c)

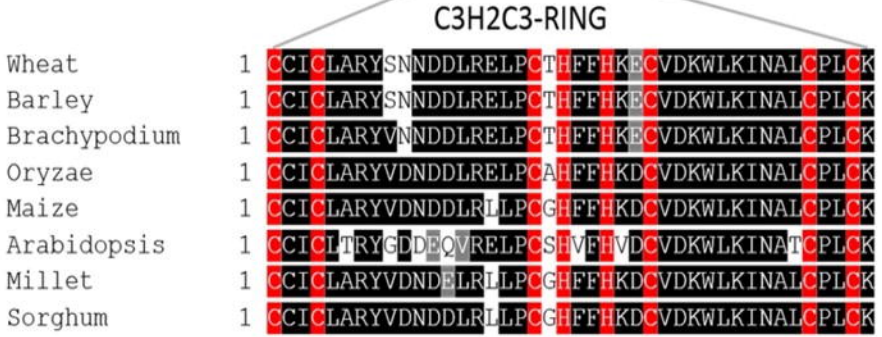

PEG101
PEG101: Zt-6
PEG101: ZtSSP2
Ratio of Anthocyanin cells to GUS cells
2.5
2.0
1.5
1.0
0.5
0.0
*
PEG101
PEG101: Zt-6
PEG101: ZtSSP2


(a)
10-1 10-2 10-3
10-1 10-2 10-3
BD-TaE3UBQ/AD-ZtSSP2
BD-TaE3UBQ126-219/AD-ZtSSP2
BD-TaE3UBQ126-219/AD-X
BD-X/AD-ZtSSP2
BD-TaE3UBQ126-219/AD-Zt10
BD-TaSSP6/AD-Zt06
-Trp-Leu
-Trp-Leu-His (+0.5mM 3AT)
-Trp-Leu-His X-αGAL
(b)
YFP
Bright field
Merged
YFPNTaE3UBQ + YFPC-ZtSSP2
YFPNTaE3UBQ + YFPC-X
YFPN -X + YFPC-ZtSSP2
Cnx6-YFPC + Cnx6-YFP
(c)
INPUT
IP (GFP-Trap)
HA-TaUBQ + + + +
GFP-ZtSSP2 + - + -
GFP - + - +
47 kD
27 kD
Anti-GFP
50 kD
Anti-HA

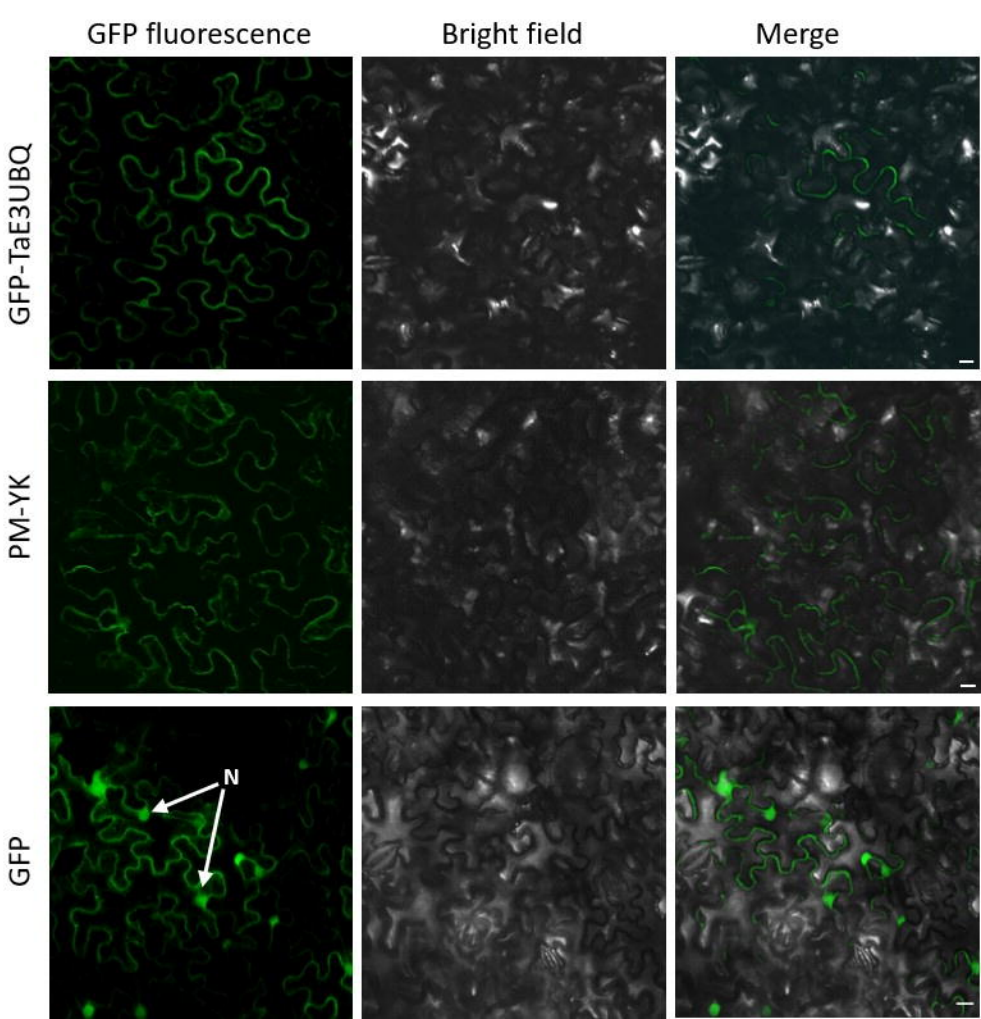
(a)

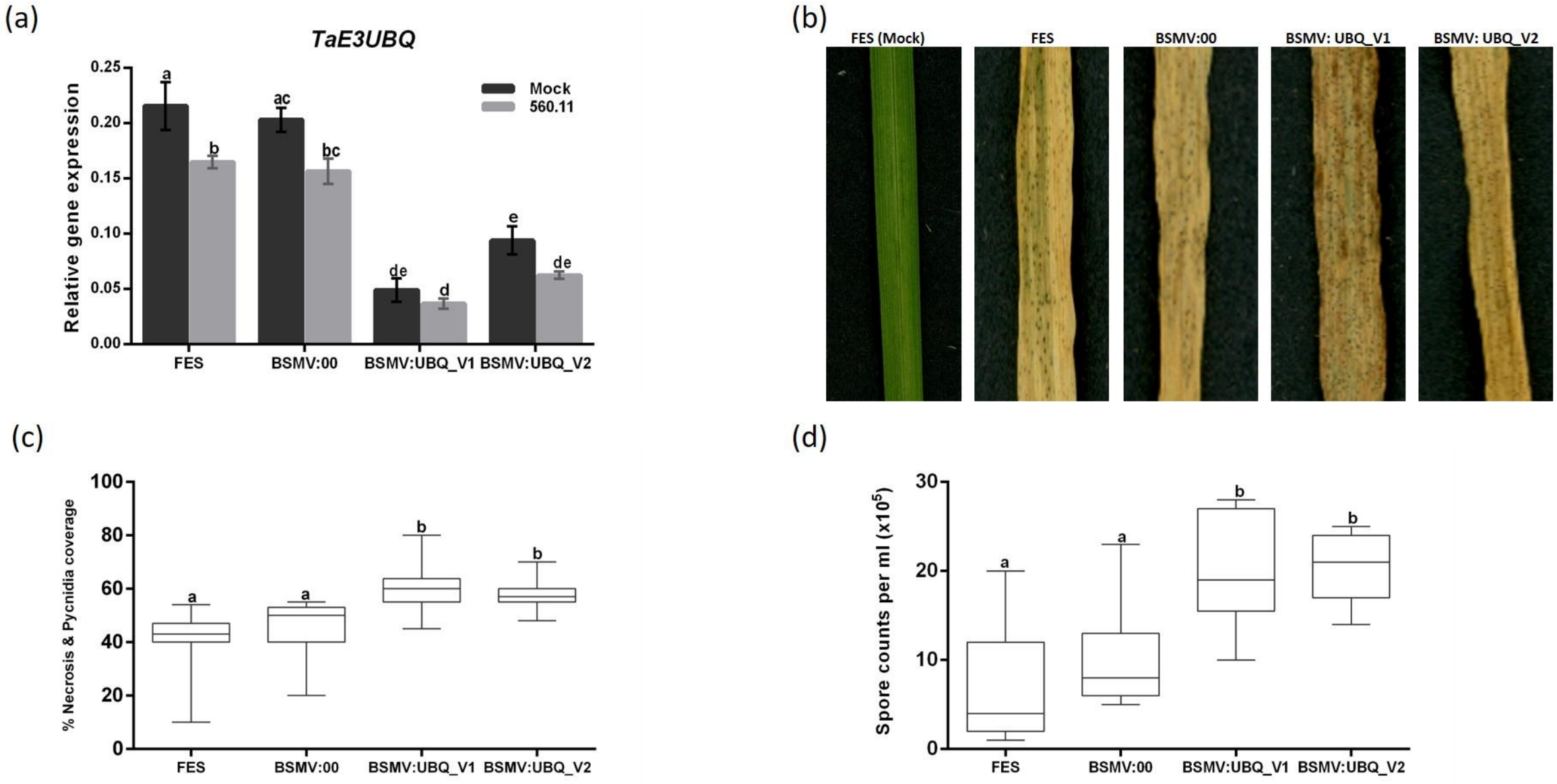

0
97 121 130 155
218 238 249 291
345
388
420
TaE3UBQ ligase
TMH TMH
TMH TMH

(b)


C3H2C3-RING
Wheat 1 CCICLARYSNNDDLRELPCTHFFHKECVDKWLKINALCPLCK
Barley 1 CCICLARYSNNDDLRELPCTHFFHKECVDKWLKINALCPLCK
Brachypodium 1 CCICLARYVNNDDLRELPCTHFFHKECVDKWLKINALCPLCK
Oryzae 1 CCICLARYVDNDDLRELPCAHFFHKDCVDKWLKINALCPLCK
Maize 1 CCICLARYVDNDDLRLLPCGHFFHKDCVDKWLKINALCPLCK
Arabidopsis 1 CCICLTRYGDDEQVRELPCSHVFHVDCVDKWLKINATCPLCK
Millet 1 CCICLARYVDNDELRLLPCGHFFHKDCVDKWLKINALCPLCK
Sorghum 1 CCICLARYVDNDDLRLLPCGHFFHKDCVDKWLKINALCPLCK

(c)


GFP fluorescence
Bright field
Merge
GFP-TaE3UBQ
PM-YK
GFP
N


(a)
TaE3UBQ
Relative gene expression
0.25
0.20
0.15
0.10
0.05
0.00
Mock
560.11
a
b
ac
bc
de
d
e
de
FES
BSMV:00
BSMV:UBQ_V1
BSMV:UBQ_V2
(b)
FES (Mock)
FES
BSMV:00
BSMV: UBQ_V1
BSMV: UBQ_V2
(c)
% Necrosis & Pycnidia coverage
100
80
60
40
20
0
a
a
b
b
FES
BSMV:00
BSMV:UBQ_V1
BSMV:UBQ_V2
(d)
Spore counts per ml (x10^5)
30
20
10
0
a
a
b
b
FES
BSMV:00
BSMV:UBQ_V1
BSMV:UBQ_V2