Ubiquitination in wheat defence against Septoria.

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Summary:

Wheat is a major food crop for much of the world, and with an ever-increasing population there is a rising demand to produce more food in a smaller area. Septoria leaf blotch mould (caused by the fungus Septoria) is a devastating foliar pathogen of wheat, which can lead to a 20% reduction in yield.
Plants have had to evolve a multitude of different defence mechanisms due to their sessile nature. Protein modifications by ubiquitination has been shown to be central to plant defence.
Virus Induced Gene Silencing (VIGS) using BSMV (Barley Stripe Mosaic Virus) has been used previously to transiently silence wheat gene expression. In this study VIGS has been used to investigate *Triticum aestivum* E2 ubiquitin conjugating (TaU) enzymes. The possible function of these TaU enzymes in the Septoria-wheat interaction was investigated after silencing the TaU enzymes in wheat and then infecting with Septoria. TaU4 silenced wheat leaves showed a delay in the onset of Septoria infection symptoms and had reduced pycnidia and spore counts when compared to the vector only control. It was concluded that TaU4 acts as a negative regulator of defence in wheat against Septoria fungal infection. The E2 function of TaU4 has also been proven through ubiquitin charging assays. Four other TaU enzymes were also studied to ascertain their role in wheat defence against Septoria.

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Declaration:

I can confirm that I did not knowingly plagiarize any of the material in this document.

Table of Contents

List of Figures:

1. Introduction

1.1. *Triticum aestivum* and Septoria fungus interaction

Triticum Aestivum (bread wheat) is one of the major food sources in many parts of the world and has been cultivated for more than 9,000 years (Kew 2014). Bread wheat is a hexaploid genome (AABBDD), which originates from the inter species hybridization of *T. urartu* (AA) and a close unknown relative of *Aegilops speltoides* (BB), this tetraploid wheat (*T. turgidum*) then hybridized with *A. tauschii* (DD) giving the hexaploid *T. aestivum* (Dvorak and Akhunov 2005; Chalupska et al 2008).

In 2013/14 world wheat production was expected to be around 708.5 million tonnes and the average amount of wheat consumed per capita is predicted to 66.9kg. This represents the largest proportion of cereal crops (total predicted cereal production is 2,497.6 million tonnes and forecasted to be 152.4kg per capita consumed). After maize, wheat has the second highest usage for animal feed in 2009 (Food and Agricultural Organisation of the United Nations, 2013a; b) therefore having importance both directly and indirectly on food production. According to the HGCA (the cereals and oilseeds division of the Agriculture and Horticulture Development Board AHDB 2012).

Septoria tritici leaf blotch is the main threat to UK wheat production, with an average potential loss in yield of 20% for untreated crop populations. It is predicted that around 70% of Europe's fungicide volume is used to treat Septoria (Ponomarenko et al 2011). Wheat is particularly vulnerable when sown upon straw debris and among early sown susceptible varieties. Septoria has become more devastating since the 1960's with the wider use of commercial, early maturing, semi dwarfing and higher yielding wheat cultivator varieties that are more susceptible to Septoria fungus (Eyal et al 1987).

Septoria leaf blotch mould is an ascomycete fungus caused by *Mycosphaerella graminicola* (teleomorph stage) in bread wheat in temperate regions. The first visible symptoms of Septoria infection are irregular brown chlorotic spots, which

expand as the infection progresses, developing into necrotic lesions on either side of the leaf. Developing with the lesions are the pycnidia, small brown raised fruiting bodies produced on the leaf at the stomata, which release the pycnidiospores when mature (Eyal 1999; Orton et al 2011). A sticky medium containing sugars and proteins is produced to protect the spores from drying out. The spores, in the sticky medium, are only exuded from pycnidia on sufficient humidity and appear through an ostiole. Septoria infection relies on water and humidity to progress (Eyal et al 1987).

The fungus spreads its spores by either wind or within rain splashes (Kema et al 1996) infecting the bottom leaves of the wheat plant and potentially going onto infect upper leaves (Eyal 1999). Over winter or when wheat crops are not present Septoria survives in plant debris as mycelium, in pycnidia and mainly as psuedothecia, which are activated to release the spores upon wetting (Ponomarenko et al 2011). It infects in temperate regions with high rainfall, ideally needing moisture for >24 hours and a temperature of 10-20^{0}C (McMullen and Adhlkarl 2009). Twenty four – forty eight hours after the spores come into contact with the leaf the Septoria hyphae infiltrate the leaf through the stomata, entering the apoplastic space, where it remains throughout its life cycle. During the early symptomless phase of infection, the first 12-14 days, the Septoria grows as a biotroph using nutrients from the apoplastic space to grow hyphae throughout the mesophyll tissue.

The Septoria genome contains few genes encoding for enzymes that break down the plant cell wall. It has been suggested that the fungus relies more on the breakdown of proteins as opposed to carbohydrate degradation to avoid detection in the biotrophic phase (Goodwin et al 2011). The next stage of the Septoria life cycle is the necrotrophic growth phase where the host's mesophyll cells collapse and die releasing nutrients, leading to leaf surface lesions (Eyal et al 1987; Palmer and Skinner 2002; Ponomarenko et al 2011). The fungal mycelium rapidly proliferate upon host cell death, with an up regulation in genes encoding for the proteins involved in energy production within the fungal cells occurring at the same time as the release of intracellular nutrients (Orton et al 2011). The cue for Septoria's switch between biotrophic to necrotrophic growth

stages is unknown as of yet. The reduction in grain yield is due to the loss of photosynthetically active tissue caused by the fungal infection during necrotrophic growth (Griton et al 2011).

Current methods of controlling the infection include the use of chemical fungicides and resistant wheat varieties (Griton et al 2011). However Septoria is becoming resistant to triazole fungicides and quinine outside inhibitors (QoIs) fungicides. Fraaije et al 2005 showed that a single point mutation in cytochrome *b* gene of the Septoria led to resistance against QoIs quickly, possibly due to QoIs only having one target for their mode of fungicide action. Applications of QoIs have now been limited to twice per season and have to be mixed with a different fungicide (sterol-demethylation inhibitors) thus hoping to slow the buildup of Septoria's resistance (Fraaije et al 2005).

The *Stb* gene family has been identified in various wheat varieties resistant to several Septoria isolates. So far 15 *Stb* (1-12 and 15-17) resistance genes, with another two possibilities (13 and 14), have been identified in wheat resistance against different isolates of Septoria (McIntosh et al 2008). However this resistance can be overcome, for example *Stb4* expressing wheat varieties (which were used for >15 years in California) have been shown to become susceptible to Septoria (Jackson et al 2000). It was also shown that *Stb4* resistance was overcome even faster (5 years) in Oregon, therefore resistance depends on both the environment and the Septoria isolates present (Cowger et al 2000). The rapid evolution of Septoria resistance is potentially due to high levels of genome plasticity within the fungus as it undergoes high frequencies of both sexual (ascopore) and asexual (conidia) reproduction (Orton et al 2011; Ponomarenko et al 2011). Wheat varieties have been bred to be resistant to different pathogens but these varieties do not yet have resistance to all the economically important diseases so the use of fungicides is still needed to prevent infection (Fraaije et al 2001).

Although many partial and fully Septoria resistant varieties of wheat have been found their method of resistance is still unknown. Currently there are no model

pathogen-plant systems similar to the Septoria-wheat infection, which makes it harder to study (Orton et al 2011).

General resistance to fungal pathogens occurs when pathogen-associated molecular patterns (PAMPs) such as eythlene-inducing xylnase (EIX) or chitins are recognised by pattern recognition receptors (PRRs) on the cell surface (Ron and Avni 2004; Kaku et al 2006). This recognition causes a cascade of events that help arm the plant for defence and is known as PAMP-triggered immunity (PTI). Some pathogens have evolved resistance, via suppression or evasion, leading to successive rounds of evolution by both the plant and pathogen that result in specific plant defences to certain pathogens known as a gene-for-gene relationship (Flor 1942; Zipfel 2008).

Specific resistance to plant pathogens at a species level involves a gene-for-gene interaction (Flor 1942) where a pathogen avirulence protein (Avr) is recognized by a plant resistance protein (R). This leads to something known as an incompatible infection, which is a hypersensitive defence response that tries to contain the fungus to a small area of the plant by killing its own tissue surrounding the pathogen (Thomma et al 2005). Susceptible plant species do not have an R gene that corresponds with the pathogen Avr gene and therefore cannot mount a defence, as it does not recognise the pathogen. There has been evidence of a gene-for-gene relationship between resistant varieties of wheat and Septoria. Brading et al (2002) studied the resistant variety *cv.* Flame and found an R gene, that they designated S*tb6*, recognised a single gene of the *M. graminicola* isolate IPO323. They also showed a strong likelihood that *cv.* Hereward also expresses the same R gene, *Stb6*, through studies of progeny of the two varieties crossed and then studying the susceptibility of the plants to Septoria infection.

1.2. Ubiquitination

1.2.1. Ubiquitination pathway and its components

Proteins are targeted to the 26S proteosome for degradation by ubiquitin protein tags. To ensure the correct proteins are degraded a set of ubiquitination enzymes work together to covalently attach a reusable polyubiquitin tag onto the protein targeted for degradation (Azevedo et al 2001; Craig et al 2009). The general theory of ubiquitination starts with the E1 ubiquitin activating enzyme. An E1-uibiquitin intermediate is formed as the E1 enzyme activates ubiquitin using ATP, then forms a stable intermediate with a thioester linkage between an E1 cysteine residue and the C terminal glycine reside of ubiquitin. E1 then transfers the ubiquitin (via transesterification) onto a cysteine residue of E2, the ubiquitin conjugating enzyme. E3 is the ubiquitin ligase enzyme. It confers the specificity for the target protein and brings the target protein into close proximity to E2 enzyme. The ubiquitin can then be transferred onto a lysine residue (via an isopeptide bond) of the target protein either directly or via an E3-ubiquitin intermediate depending on the class of E3 ligase. Cycles of these steps follow to transfer more ubiquitin molecules (Kannouche and Lehmann 2004) onto Lysine48 of the previous ubiquitin, to form the polyubiquitin tag of 4 or more ubiquitin proteins that signals to the 26S proteosome to degrade the protein (Thrower et al 2000). These steps require an additional enzyme, E4, to generate the polyubiquitin chain (Koegl et al 1999).

As well as adding ubiquitin, there are also enzymes that control the removal of ubiquitin known as deubiquiting enzymes (DUBs). These enzymes act as cysteine proteases that cleave ubiquitin from the target protein or from ubiquitin chains by hydrolyzing the amide bond after the N terminal glycine of ubiquitin (Glickman and Ciechanover 2001).

Ubiquitination does not always lead to the target protein being degraded by the 26S proteosome; differing chain lengths and attachments can signal for other possible outcomes. These include endocytosis, signal transduction (Hicke 2001), DNA damage repair (Kannouche and Lehmann 2004), degradation of proteins in

the lysosyme (Chastagner et al 2006). The number and specificity of the ubiquitin enzymes increases from E1 to E2 to E3, for example in *Arabidopsis thaliana* there are 2 E1, 41 E2 and 1200 E3 enzymes. This allows for accurate recognition and therefore ubiquitination of the target protein. (Aravind and Koonin 2000; Azevedo et al 2001; Fang and Weissman 2004; Craig et al 2009).

There are 7 lysine residues in ubiquitin (Lys^{6}, Lys^{11}, Lys^{27}, Lys^{29}, Lys^{33}, Lys^{48}, and Lys^{63}) and so 7 different linkages that the ubiquitin chains can form. The model above is not always followed exactly. For instance it has been shown that some E2 enzymes can build pre-formed ubiquitin chains before tagging the substrate. An example of this is Ube2g2 enzyme (from mice and identical to human Ube2g2), which works in pairs to generate an ubiquitin chain on one of the Ube2g2 with the help of E3 ligase gp78c (Li et al 2007).

Another model variation is that different E2 enzymes may be needed to attach the first ubiquitin and to attach subsequent ubiquitin molecules (Windheim et al 2007). Other E2 enzymes can function in both the chain initiation and elongation, such as Cdc34 that multiubiquitinates Sic1 leading to Sic1 degradation and passage from G_1 to S phase in the cell cycle (Verma et al 1997).

Some E2 enzymes are also specific in the chain linkage they form, for instance UBE2S only elongates onto lysine11 of the previous ubiquitin (after chain initiation by UbcH10) (Williamson et al 2009) and Ubc13-Uev1a only elongates onto lysine63 of the previous ubiquitin (Windheim et al 2007).

The specific chain linkages can be independent of the E3 ligase partner. It has been suggested that the E2 enzymes can determine the linkage by orientating the ubiquitin on the substrate so that the favoured lysine is aimed towards the active site where the ubiquitin to be attached is located (Petroski and Deshaies 2005). These E2 enzyme chain linkage preferences generally work in partnership with RING E3 ligases, which do not make an ubiquitin-E3 intermediate and instead hold the substrate and E2 enzyme in close proximity to allow transfer of the ubiquitin. HECT E3 ligases make a thioester bond between the active site cysteine residue and the N terminal glycine of ubiquitin, and therefore can determine the specificity of the ubiquitin chain linkage (Wang et al 2006).

1.2.2. Ubiquitination in plant defence

Ubiquitination has been noted as important in plant defence. Within the cereal family there are a number of E3 ligases that have been studied. So far ubiquitination processes within wheat defence have yet to be studied.

Takai et al (2002) found a RING-finger E3 ligase enzyme within rice (EL5) that was activated by elicitor treatment. Fragments of chitin (N-acetylchitooligosaccharides) from phytopathogenic fungus backbone (for example from rice blast fungus) acted as the elicitor. They also identified a potential E2 enzyme (OsUBC5b) from rice that became upregulated after elicitor treatment and could ubiquitinate EL5. EL5 belongs to the ATL family of RING-finger E3 ligases. ATL family genes all share a RING-H2 finger domain and an N-terminal transmembrane domain (Fang and Weissman 2004). Other members of the ATL family have also been implicated in plant defence within rice and other plant species. OsBIRF1, another rice RING-finger E3 ligase of the ATL family, has also been implicated in rice defence responses. Liu et al (2008) studied the infection of rice blast fungus upon two strains of rice, H8R and H8S, which are resistant and susceptible to the fungus respectively. Within the resistant strain (H8R) an incompatible interaction occurred, accompanied with an increase in the gene expression of *OsBIRF1*. They also overexpressed *OsBIRF1* in tobacco and showed increased resistance to tobacco mosaic virus (TMV) and *Pseudomonas syringae.*

Avr9/Cf-9 rapidly elicited (ACRE) genes have been shown to be rapidly upregulated in tomato plants that express the *Cf-9* gene and so are resistant to *Cladosporium fulvum* infection via a gene-for-gene interaction (*Avr9/Cf-9*) (Durrant et al 2000). Durrant et al (2000) studied the expression profile of many proteins after Avr9 treatment and found 13 ACRE genes that had been upregulated. Within these *ACRE* genes was *ACRE-132*, a member of the ATL family of RING-H2 finger domain E3 ligases. *C. fulvum* infection upon tomato is similar to that of Septoria upon wheat as they both stay in the apoplastic space during infection and never infect the cell (Thomma et al 2005; Otron et al 2011).

SGT1 was identified within yeast as an associating member of the SCF E3 ligase complex by interactions with Skp1 (Kitagawa et al 1999). Azevedo et al (2002) silenced *hvSGT1* in barley that expressed the R genes *Mla1* and *Mla6* and then infected the silenced cells with powdery mildew expressing the avirulence gene that corresponded to the resistance R gene (*AvrMla1* and *AvrMla6* respectively). They found silencing of *hvSGT1* in *Mla1* expressing barley had no effect upon the infection process. For *hvSGT1* silenced *Mla6* expressing barley there was a reduction in the resistance, with around 63% of epidermal cells becoming infected with powdery mildew. This indicates that *hvSGT1* is needed for the gene-for-gene resistance mechanism between the R gene *Mla6* in barley and the avirlunce gene Avr*Mla6* expressed by strains of powdery mildew. *hvSGT1* interacts with *hvRAR1* (which acts downstream of Avr fungus protein recognition by R plant protein). This indicates that hvSGT1 is involved in the gene-for-gene interaction as it passes the signal on from hvRAR1 and this signal pathway involves ubiquitination using SCF complex E3 ligase complex. Peart et al (2002) also silenced SGT1 in *Nicotiana benthamiana* and found that it was involved in general host defence. Silencing caused a reduction in the resistance to pathogens that do not usually infect *N. benthamiana*, such as *Xanthomonas axonopodis*, which infects pepper plants.

Ubiquitination is also involved in attenuating plant defences to avoid energy wastage. For instance Lu et al (2011) studied FLAGELLIN SENSING 2 (FLS2), which recognises flagellin of bacteria (Gomez-Gomez and Boller 2000). They found that after recognition of flg22 (22 amino acids from the N terminal of flagellin shown to trigger plant immunity (Felix et al 1999)) two ubiquitin ligase proteins (PUB12 and PUB13) worked together to ubiquitinate FLS2 and target it for degradation by the 26S proteosome therefore attenuating the defence response.

Silencing has been used to study the effects of ubiquitin enzymes in plant defence. A study using *N. tabacum* that expressed *Cf-9* (from tomato and recognizes Avr9 protein from *C. fulvum*) focused on CMPG1, which is a homolog of ACRE74 from tomato. ACRE74 is a U box E3 ligase. They silenced the *CMPG1* and infiltrated the silenced *N. tabacum* with Avr9 peptide. The silenced plants

showed a reduced incompatible infection, HR and so resistance. Overexpressing CMPG1 showed the opposite effects, with increased HR and higher levels of resistance (Gonzalez-Lamothe et al 2006).

1.3. Techniques for genetic studies in wheat

Studies of wheat are made harder due to its large (16,000Mb) hexaploid genome (Flavell et al 1974). The hexaploid genome is made up from three genomes designated A, B and D that are all closely related. For wheat genes there are three or n*three copies. This causes a problem with functional redundancy as many of these homologous genes are expressed therefore a mutation must be made across each of the three genomes to allow the study of the gene function. (Mochida et al 2003; Travella et al 2006).

Reverse genetic techniques are used when the gene sequence is known but the protein function is unknown. Since the rise of sequenced genomes, reverse genetics has become a useful tool in molecular biology. It usually involves modifying the gene or expression of the gene, which causes a phenotype change. Reverse genetic techniques include silencing gene expression (e.g. virus induced gene silencing (VIGS)), insertion mutations into the gene sequence (e.g. T-DNA or transposons) or chemical mutagenesis (using ethylmethane sulphonate or ethyl nitrosourea) (Gilchrist and Haughn 2010).

1.3.1. Virus Induced Gene Silencing

VIGS uses the posttranscriptional gene silencing (PTGS) system used by plants to defend themselves against viral pathogens. It allows the silencing of homologous genes (Travella et al 2006). At the nucleotide sequence level wheat genes can share up to 99% similarity between homologous genes across the three genomes (Kimbara et al 2004). VIGS can also silence genes that have >85% sequence homology, allowing silencing across the three wheat genomes (Thomas et al

2001; Holzberg et al 2002). When the virus infects the cell it releases its single stranded RNA into the cytoplasm. The virus then replicates its RNA using a viral encoded, RNA-dependent RNA polymerase, producing sense and anti-sense RNA. The sense and anti-sense RNA may hybridise to form a double stranded viral RNA molecule, which is recognised by the plant protein DICER (an RNaseIII-type enzyme) and cleaved into small interfering RNA (siRNA), 21-23 nucleotides long (Hamilton et al, 2002: Lu et al, 2003). These siRNA are then incorporated into the plant RNAi silencing complex (RISC complex). If the RISC complex comes into contact with any mRNA that are complementary to the siRNA associated with the complex it cleaves these mRNA, leading to mRNA silencing of the viral genes. This slows viral replication and can give the plant immunity to any following infections from this virus. (Voinnet et al 2000; Vance and Vaucheret 2001; Waterhouse and Helliwell 2002; Wang and Metzlaff 2005).

VIGS studies can be used to discover the function of a plant protein whose DNA sequence is known by silencing and then studying for any effects (Baulcombe 1999). By cloning a small fragment of the selected plant gene into the virus vector the plants own viral defence mechanisms can be used to transiently silence endogenous mRNA (Tai et al 2005). Cloning additional fragments into the viral RNA does not affect the virus infectivity (Waterhouse and Helliwell 2003). As the modified virus spreads throughout the plant so does the endogenous mRNA silencing (Voinnet et al 2000). Bruun-Rasmussen et al (2007) showed that the fragment inserted into the virus was better maintained and more abundant with smaller inserts, particularly if the insert is around 120 nucleotides. The vector chosen must be from a virus that naturally infects the host plant. Barley Stripe Mosaic Virus (BSMV) naturally infects certain monocot species such as barley, wheat, oats and maize (McKinny and Greeley 1965). Haupt et al (2001) showed that BSMV can spread across barley leaves using GFP expressing BSMV, this indicates that BSMV can be used to silence genes across the leaf.

Before silencing wheat with the modified BSMV vector, the BSMV vector is amplified by infection into *N. benthamiana*. It offers a quick, inexpensive and easy way to increase the amount of modified virus for infection into lots of wheat plants. The insert (of the gene to be silenced) has been shown to be stable in *N.*

benthamiana and levels of silencing from the *N. benthamiana* sap were comparable to primary infection of wheat with BSMV (Yuan et al 2011).

VIGS can be more useful than gene knockouts, for example insertional mutagenesis, for a number of reasons. Firstly gene expression silencing does not occur until the plant is infected with the virus, therefore any genes involved in cell growth or development can be studied. It also does not cause full gene expression silencing as low amounts of residual mRNA are left therefore essential genes may be studied without being lethal to the plant. This is also a negative point as the phenotype seen after silencing may be due to the residual levels of gene expression (Lu et al 2003; Travella et al 2006). The viral construct can be generated quickly and the phenotype of PDS silencing through VIGS appears 1-2 weeks post infection. As only a small fragment of the gene to be silenced is cloned into the BSMV vector full knowledge of the target gene is not required (Scofield and Nelson 2009).

1.4. Literature Review Conclusion

With wheat crops being such an important food source throughout the world it is important to learn more about factors that lead to reductions in the yield. Septoria fungus can cause a potential reduction in yield of up to 20% (HGCA, 2012). This means studying Septoria infection is very important. Unfortunately due to wheat's hexaploid genome generating transgenic plants, therefore performing reverse genetics, is not as straight forward as in the model plant *A. thaliana* (Mochida et al 2003; Travella et al 2006). VIGS can be performed relatively quickly and leads to the transient silencing of gene expression allowing reverse genetic study of known gene sequences (Baulcombe 1999). The BSMV vector has proven successful for silencing within wheat using visible phenotype caused by PDS silencing (Tai et al 2005) therefore this system can be utilised for the revere genetic study of wheat genes. It has been shown in other pathogen plant interactions that the ubiquitin system is involved in the plants defence response. E3 ligases are the most studied within this area as they offer the

highest specificity to the target protein. Ubiquitination can signal a target protein to many different outcomes, not just degradation, by attachment at different sites on the ubiquitin, differing chain lengths or differing chain configurations. This offers a wide range of possibilities for ubiquitination and ubiquitin enzymes to control many cellular pathways including cell defence (Hicke 2001; Kannouche and Lehmann 2004; Chastagner et al 2006). Hence the study of ubiquitination within *S*eptoria-wheat infection could help to find a method of resistance.

This study used VIGS to investigate the role of *Triticum aestivum* ubiquitin conjugating enzyme 4 (TaU4) has in Septoria infection upon wheat. Using VIGS technique to transiently silence TaU4 expression and assessing the changes in phenotype severity this causes led to finding that TaU4 is potentially involved in the negatively regulating plant defence. After investigation of the E2 function of TaU4 it was found that it was indeed an E2 enzyme that can bind to ubiquitin (via a thioester bond), which has been previously activated by an E1 enzyme. TaU4 was also found to localize to both the nucleus and the cytoplasm using confocal microscopy and YFP:TaU4 construct. The localization can indicate potential E3 enzyme interacting partners and target substrates. 4 other TaU enzymes were also found to be potentially involved in the Septoria-wheat interaction, TaU1, TaU2, TaU3 and TaU5 (Lee et al unpublished). Using VIGS to transiently silence the 4 TaU enzymes their possible roles in wheat defence against Septoria were studied. This led to other promising avenues of investigation being discovered, which would need to be studied in further detail to confirm any involvement in wheat defence against Septoria.

1.4.1. Aims

Other studies have shown ubiquitination plays a role in plant defence against pathogens. Therefore the role of ubiquitination in wheat defence against the devastating fungal pathogen Septoria was investigated with the aim to find a wheat E2 enzyme that showed marked Septoria infection changes after being silenced (VIGS) in the infected wheat leaves.

1.4.2. Objectives

To establish that *TaU4* has a role in wheat defence against Septoria, using the VIGS technique.

To establish that TaU4 is an E2 enzyme that can become ubiquitinated upon its active site cysteine via a thioester bond.

To investigate four other TaU enzymes from wheat and their possible roles within wheat defence against Septoria.

2. Methods

2.1. RNA extraction

75mg of frozen leaf tissue was ground to a powder and then 750μl of trizol (Zymo Research, Irvine, USA) was added before vortexing to mix thoroughly. RNA was extracted using Direct-zol™ RNA miniprep kit (Zymo Research) including the In-column DNase I digestion and eluted in 30μl of DNase/RNase free water. RNA concentration was then measured using Nanodrop ND-1000 spectrophotometer (Labtech, Uckfield, England). The RNA was stored at -80^{O}C.

2.2. cDNA synthesis

1-4ng of RNA was used, with sterile distilled water added to make a final volume of 10μl. To amplify the mRNA 1μl of oligo dT (10mM) (VWR, Radnor, USA) was added to the RNA mixture and then heated at 65^{O}C for 5 minutes before placing on ice. Then 4μl of 5x strand buffer (Invitrogen, Grand Island, USA), 2μl of DTT (Invitrogen), 1μl of dNTP (10mM each) (VWR) and 1μl of RNase OUT (Invitrogen) were added to the RNA mixture. This was then heated to 42^{O}C for 2 minutes before the addition of 1μl of Superscript II (Invitrogen). The mixture was then heated at 42^{O}C for 50 minutes and then 70^{O}C for 15 minutes. The cDNA was stored at -20^{O}C.

2.3. PCR

All primers used, the expected band size and the melting temperature for each primer pair is listed in the appendix. All primers were ordered from Eurofins (Huntsville, USA) and used at a concentration of 10pmol/μl.

2.4. Polymerase chain reaction (PCR)

2.4.1. Taq polymerase PCR

Each 20μl PCR reaction comprised of 10μl of reddy mix (Bioline, London, England), 1μl forward primer, 1μl reverse primer, 1μl of cDNA* and 7μl of sterile distilled water, which were spun down before PCR. These were then run on a PCR program (TC-3000G Techne machine) –

$94^{O}C$ for 5 minutes,

$94^{O}C$ for 30 seconds,
$X^{O}C$ for 30 seconds,
} 25-35 cycles

$72^{O}C$ for Y seconds,

$72^{O}C$ for 5 minutes,

Hold at $12^{O}C$.

X = ~3 degrees lower than the primers melting temperature.

Y = 1 minute per 1Kb of gene to be amplified.

To test PCR primers they were firstly run on a gradient PCR with the annealing temperature set at ~-6 to $+2^{O}C$ degrees of the Tm. The highest temperature that showed a band was selected as the annealing temperature.

The PCR products were run on an agarose gel (gel electrophoresis) to see the results.

*For colony PCR a single colony was taken and resuspended in 20μl of sterile distilled water, 1μl of this then replaced the 1μl cDNA used.

2.4.2. Q5 polymerase proof reading PCR

Each 50µl PCR reaction comprised of 10µl of 5X Q5 reaction buffer (NEB, Ipswich, USA), 1µl dNTP's (10mM), 2.5µl forward primer, 2.5µl reverse primer, 2.5µl of cDNA, 0.5µl Q5 high-fidelity DNA polymerase (NEB) and 31µl of sterile distilled water, which were then spun down before PCR. These were then run on a PCR program -

$98^{O}C$ for 30 seconds,

$98^{O}C$ for 10 seconds,

$X^{O}C$ for 30 seconds,

} 25-35 cycles

$72^{O}C$ for Y seconds,

$72^{O}C$ for 2 minutes,

Hold at $12^{O}C$.

X = ~3 degrees lower than the primers melting temperature.

Y = 30 seconds per 1Kb of gene to be amplified.

The PCR products were run on an agarose gel (gel electrophoresis) to see the results.

2.4.3. Site directed mutagenesis

Each 40µl PCR reaction comprised of 8µl of 5X Q5 reaction buffer, 0.8µl dNTP's (10mM), 2µl forward primer, 2µl reverse primer, 1µl of plasmid (10ng/µl), 0.4µl Q5 high-fidelity DNA polymerase and 25.8µl of sterile distilled water which were then spun down before PCR. These were then run on a PCR program -

98^{O}C for 30 seconds,

98^{O}C for 10 seconds,

X^{O}C for 30 seconds,

72^{O}C for Y seconds,

} 25-35 cycles

72^{O}C for 5 minutes,

Hold at 12^{O}C.

X = ~3 degrees lower than the primers melting temperature.

Y = 30 seconds per 1Kb of gene to be amplified.

Next, to digest the methlyated DNA template, 1µl of DpnI (NEB) was added directly to the 40µl PCR reaction, vortexed briefly and spun down before incubation at 37^{O}C for 2 hours. One hour into the incubation the reactions were vortexed and spun down and transferred to a new 0.2ml PCR tube (Starlab, Milton Keynes, USA). After the 2 hours the reaction was heated at 80^{O}C for 20 minutes to denature the DpnI enzyme. The mutated plasmid was then transformed into DH5α *E. coli* cells (2.7. Transformation).

2.4.4. Real time PCR

For a 20µl, 10µl of SYBR green (Sigma Aldrich, St. Louis, USA), 1µl forward primer, 1µl reverse primer, 1µl of cDNA and 7µl of sterile distilled water were added together before being spun down. This was then run on a PCR program (Corbett real time PCR machine) –

94^{O}C for 5 minutes,

94^{O}C for 30 seconds,
X^{O}C for 30 seconds,
} 40 cycles

72^{O}C for Y seconds,

72^{O}C for 5 minutes,

Hold at 12^{O}C.

X = ~3 degrees lower than the primers melting temperature.

Y = 30 seconds per 1Kb of gene to be amplified.

The cycle threshold (Ct) values were then normalised to the housekeeping reference gene.

To test the real time PCR primers serial dilutions of cDNA (x1, x2 and x4) were performed and the Ct values compared.

2.5. Gel electrophoresis

Different percentage agarose gels were made depending on the size of the fragment to be visualized. The gels were between 0.8-1.2%, with the higher concentrations being used for the smaller fragments. Per 100ml of 1x TAE buffer (Biorad, West Berkeley, USA) between 0.8-1.2g of agarose (Melford, Ipswich, England) was added and then heated in a microwave until the agarose had dissolved. Per 100 ml of solution 0.75μl of ethidium bromide (Fischer Scientific, Waltham, USA) was then added. This was left to set for 30 minutes or until set. The gel tank contained 1x TAE buffer, which filled the tank to above the level of the gel. Five μls of the appropriate hyperladder (either 50bp or 1Kb depending

on size of fragment) (Bioline) was pipetted into the first well. Subsequent wells were then filled with 9μl of the PCR reaction*. The gel tank was run at ~100 volts until the dye was over half way through the gel. The fragments were visualized under a UV using a Gene Flash machine and Quantity One program on the computer.

*For PCR reactions using Q5 5μl of 10x loading dye was added before loading into the gel wells.

2.6. Gel extraction

The fragment to be extracted was first run using the method as described in Q5 polymerase proof reading PCR and Gel electrophoresis (2.4.2 and 3.5 respectively). Then the gel was placed on a UV light box and the excess gel removed using a blade. Using the UV light the band was excised and put into a pre weighed 1.5ml epindorf tube. The tube was then reweighed to get the weight of the gel. The gel extracted was done following the instructions in the QIAquick gel extraction kit (Qiagen, Limburg, Netherlands). At the elution stage 30μl of sterile distilled water was used.

2.6. Cloning

2.6.1. Vector ligation

D-TOPO (gateway vector)

To 1μl of D-TOPO (Invitrogen) 5μl of PCR product was added and shook gently. This was then left to stand for 30 minutes at room temperature and put onto ice before transformation.

2.6.2. Miniprep

10ml of LB with appropriate antibiotic (Melford) (50μg/ml of kanamycin for D-TOPO, pEarleyGate104 and BSMV or 50μg/ml of carbenicillin for pDEST15) and bacterial colony was grown overnight at 37^{O}C (*E. coli*) or 28^{O}C (*Agrobacterium*). The culture was spun down in the morning for 10 minutes, at 5,000rpm and 4^{O}C and the supernatent discarded leaving the bacterial pellet. The vector was then isolated from the bacteria using QIAprep^{R} spin miniprep kit (Qiagen) eluting in 30μl of DNase free water. The concentration of vector was measured using a Nanodrop ND-1000 spectrophotometer and stored at -20^{O}C.

2.6.3. LR reaction (into gateway donor vector)

To transfer a fragment between a donor vector and a destination vector, 0.5μl of donor vector (50-150ng), 0.5μl of destination vector (150ng) and 1μl of TE buffer (pH 8.0) were added together. LR clonase II enzyme (Invitrogen) was thawed on ice for 2 minutes and then vortexed for 2 seconds twice. Zero point five μls of the enzyme was then added to the vector mixture and vortexed twice briefly before a brief centrifugation. The mixture was then incubated at 25^{O}C for 1 hour. To stop the reaction 0.5μl of Proteinase K solution (Invitrogen) was added, vortexed briefly and then incubated at 37^{O}C for 10 minutes. This was then placed on ice before transformation.

2.6.4. BSMVγ

First the BSMVγ vector was digested with Apa1 (Promega, Madison, USA). 2μl of 10x Promega buffer A, 2μl of acetylated BSA (1/10), 1μg of BSMVγ and up to 19.5μl were mixed together by pipetting. 0.5μl of Apa1 enzyme (Promega) was

then added to the reaction mixture and incubated for 4 hours at 37^{0}C. To inactivate the Apa1 enzyme it was incubated at 65^{0}C for 15 minutes.

Next two reactions were set up at the same time, a vector mixture and a fragment mixture. The vector mixture consisted of 1μl of fragment DNA, 0.5μl of dATP (100mM), 1μl of BSA (1/10 diluted), 1μl of 10x T4 buffer, 2μl of T4 DNA polymerase (1/10 diluted) and 4.5μl of sterile distilled water. The digestion mixture consisted of 4μl of digested BMSVγ mixture, 1μl of dTTP (100mM), 2μl of BSA (1/10 diluted), 2μl of 10x T4 buffer, 2μl of T4 DNA polymerase (1/10 diluted) and 9μl of sterile distilled water. Both the vector mixture and the digestion mixture were incubated at room temperature for 30 minutes before inactivation of the enzyme at 75^{0}C for 15 minutes. 2μl of the vector mixture was then added to the 20μl of digestion mixture before being incubated at 65^{0}C for 2 minutes and then incubated at room temperature for 10 minutes. This mixture was then transformed into chemically competent DH5α *E. coli*.

2.7. Transformation

2.7.1. *E. coli* (DH5alpha and BL21)

200μl of chemically competent cells (DH5α or BL21) were thawed on ice before the addition of 1μl of the vector (either from D-TOPO, LR reaction or BSMV cloning). The cells were then heat shocked at 42^{0}C for 30 seconds before placing on ice. 250μl of S.O.C. medium (Super Optimal broth with Catabolite repression) was added and incubated with shaking at 37^{0}C for 1 hour. The cells were then spread onto LB agar plates containing the appropriate antibiotic for the vector (50μg/ml of kanamycin for D-TOPO, pEarleyGate104 and BSMV or 50μg/ml of carbenicillin for pDEST15) and left overnight to incubate at 37^{0}C.

2.7.2. Agrobacterium

The *Agrobacterium* strain GV3101, pMP90 that is resistant to both rifampicin (25μg/ml) and gentimycin (25μg/ml) was used for all *Agrobacterium* transformations.

200μl of chemically competent cells were thawed on ice before the addition of 1μg of vector. This was then incubated for 5 minutes on ice, liquid nitrogen for 5 minutes and then 37^{0}C for 5 minutes. To this mixture 1 ml of LB was added before being incubated for 2 hours at 37^{0}C. The cells were then spread out onto LB agar plates containing the appropriate antibiotic for the vector (50 μg/ml of kanamycin for BSMV and pEarleygate104) and left to incubate for 48 hours at 28^{0}C.

2.8. Protein expression

E. coli strain BL21 containing the gene for the protein to be expressed was grown in a 10ml LB culture containing the appropriate antibiotic (50μg/ml of carbenicillin for pDEST15) at 37^{0}C overnight with shaking. To test for the incubation time that allowed the best protein expression, 0.5ml of the overnight culture was added to 50ml of LB and grown with shaking at 37^{0}C until the optical density (O.D.) at 600 wavelength of the culture reached between 0.6-0.8 (against LB with the antibiotic added). Once the culture reached 0.6-0.8 O.D. 600 two 1ml samples were taken and put onto ice. 50μl of IPTG (Fischer Scientific) (1M) was then added to the culture to induce protein expression from the inducible pDEST15 vector.

Samples were then taken at 1, 2 and 3 hours after induction with IPTG. The O.D. 600 was measured each time and the amount of sample collected was calculated so that the same amount of bacterial cells were collected. The samples were then spun down for 2 minutes at 15,000g. The supernatent was discarded. The two lots of collected samples were the total protein fraction and the

soluble/insoluble protein fraction. The total protein fraction was resuspended in 60µl of sterile distilled water by pipetting and 20µl of 4x SDS PAGE loading buffer was then added. The insoluble/soluble protein fraction was resuspended in 60µl of bug buster (Novagen, Billerica, USA) (with proteinase K (Roche, Basal, Sweden) added 1 tablet per 10ml of bug buster). This was then spun down for 1 minute at 13,000rpm in a microcentrifuge (Thermo Scientific Waltham, USA, Heraeus Pico 17 centrifuge). The supernatent was pipetted out into a fresh tube and 20µl of 4x SDS loading buffer, this is the soluble protein fraction. The remaining pellet was resuspended in 60µl of deionised water by pipetting and 20µl of 4x SDS loading buffer was added. All the samples were heated at 95^{O}C for 5 minutes to destroy the tertiary structure before loading onto a SDS-PAGE gel to separate the proteins and then visualized by either western blotting or comassie staining. This was then repeated with the appropriate protein induction timing. At the end of the induction the entire culture was spun down and the supernatent poured away. The remaining bacterial pellet was stored at -20^{O}C in pre weighed centrifuge tube.

2.9. Protein purification

The bacterial pellet from the protein expression was weighed and 5ml per gram of bug buster (with proteinase K, 1 tablet per 10ml of bug buster) was added. This was incubated at room temperature on a horizontal shaker for 15-20 minutes. Afterwards the protein mixture was centrifuged for 15 minutes at 30,000g. For crude extraction the supernatent was then moved into a fresh 1.5ml epindorf tube using a syringe with a needle attached to avoid getting any of the bacterial pellet in the supernatent and then filtered through a 0.45µM filter, this was the crude extract.

The GSTrap 4B (GE healthcare, Little Chalfont, USA) 1ml column was set up and 5ml of pure filtered water was run through the column at 2ml/min. Five ml of the binding buffer was run through the column at 1ml/min. The crude extract of protein was run through the column at 0.2ml/min to ensure the GST protein

would bind. The flow through from the crude extract was collected. The column was washed with 5-10ml of binding buffer at a flow rate of 1ml/min. Then 10ml of elution buffer was run through the column at a flow rate of 1ml/min. The flow through of this was collected in 1.5ml epindorf tubes in 10 x 1ml fractions.

100µl of each 1ml fraction was collected and 33µl of 4x SDS loading buffer was added. These fraction samples were then heated at 95^{O}C for 5 minutes to remove tertiary structure and loaded onto a SDS PAGE gel for protein separation. This was then visualized by either western blotting or comassie staining to see which fractions contained the most protein. The protein fractions were then stored at -80^{O}C before concentration and buffer changing.

To concentrate the proteins and remove the buffer containing glutathione 500µl of the protein fractions containing the protein was pipetted into a centrifugal filter column (Millipore, Billerica, USA). This was then centrifuged at 13,000rpm for 5 minutes at 0^{O}C and repeated until the entire protein fraction was run through the column. To remove the glutathione, 500µl of the elution buffer without glutathione was loaded onto the column and centrifuged at 15,000g for 5 minutes at 0^{O}C and repeated. Finally 400µl of the ubiquitin assay buffer was loaded onto the column and the column flipped upside down in the collection tube. This was then centrifuged at 15,000g for 5 minutes at 0^{O}C. The collected protein in the ubiquitin assay buffer was then aliquoted and stored at -80^{O}C.

2.9. Ubiquitin E2 assay

The thioester reaction contained 4µl of E1 ubiquitin activating enzyme (Enzo Lifesciences, Exeter, USA) (0.5µg/µl), 2/6.8/21.9µl of E2 ubiquitin activating enzyme UBCH5b (Enzo Lifesciences)/TaU4/TaU4^{C88S}, 3µl of 10x ubiquitin assay buffer, 6.5µl of ubiquitin (1µg/µl) and 14.5µl of sterile distilled water. The controls for this assay were –E1, -ATP, -ubiquitin, +ve E2 (UBCH5b) and the mutant TaU4^{C88S} and the levels of sterile distilled water were amended to ensure the assay was 30µl in total volume.

The assays were then incubated at 30^{O}C for 5 minutes before being transferred to ice. 30µl of 2x non-reducing SDS loading buffer was added to each of the assays and the controls. The assay samples were then split into a non reduced and reduced sample as follows: for the reduced sample 22.5µl was pipetted into a new 1.5ml epindorf tube. To the reduced samples 1.2µl of DTT (1M) was added and the samples were incubated at 95^{O}C for 5 minutes. The remaining 37.5µl of assay sample was incubated for 15 minutes at 30^{O}C. 2 x 7µl of each the reduced and non reduced samples were run on separate SDS PAGE gels to avoid reducing the non reduced sample. A western blot was performed to visualize the proteins using anti-ubiquitin (1:5,000, Sigma Aldrich) and anti-GST (1:5,000, Sigma Aldrich) on separate blots.

2.10. Protein extraction

1g of leaf tissue was collected and frozen on liquid nitrogen. At 4^{O}C the tissue sample was ground to a powder before the addition of 1.6ml of protein extraction buffer. This was ground until homogenous with the thick consistency. A pinch of 1.5w/v of PVPP (Sigma Aldrich) was added and mixed in by grinding to inhibit any phenolics in the plant tissue. The mixture was then spun down for 15 minutes at 9,600g, 4^{O}C and the supernatent transferred to a new 1.5ml epindorf tube. The pellet was discarded. Four times SDS page loading buffer was mixed in by pipetting and then heated for 5 minutes at 98^{O}C before being loaded onto a SDS PAGE gel for protein separation.

2.11. SDS PAGE gel

Gels of 12% were made based on the molecular weight of the proteins (10-70kDa for a 12% gel). For 10ml of 12% gel 4ml H_2O, 3.3ml 30% acrylamide (Sigma Aldrich), 2.5ml Tris.HCl (1.5M pH 6.8), 100µl SDS (10%), 4µl TEMED (Fischer Scientific) and 100µl of APS (10%, Sigma Aldrich) were added together

for the running gel. 7ml of this mixture was pipetted into a 15mm gel mould and 100% isopropanoyl (Fischer Scientific) was syringed on top to create a level gel. This was left for 30 minutes to set before pouring off the isopropanyl and making the stacking gel. For 5ml of stacking gel 3.4ml H_2O, 830µl acrylamide, 630µl Tris.HCl (1M pH 6.8), 50µl SDS (10%), 5µl TEMED and 50µl of APS (10%) were added together. 3ml of the stacking gel mixture was pipetted on top of the running gel and a 15 well comb was placed into the mould. This was allowed to set for 30 minutes. The comb was removed from the gel and the gel placed into the gel tank with 1x running buffer. Up to 30µl of protein was loaded into the wells with the first lane loaded with 5µl of PAGE ruler protein ladder (Thermo Scientific).

The gel tank was run at 60 volts for 3 hours or until the loading dye reached the bottom of the gel. Either comassie staining or western blotting then visualized the protein bands.

2.12. Comassie staining

The protein gel was shook in comassie dye on a horizontal shaker for 30 minutes before destain was added. The gel in destain was left overnight shaking and a photo taken of the gel in the morning.

2.13. Western blotting

The PVDF membrane was activated by soaking and shaking in methanol for 5 minutes. The membrane was then soaked and shook in 1x transfer buffer for 5 minutes before assembly of the transfer. Each of the components for the transfer was first soaked in 1x transfer buffer for 5 minutes. Starting from the black side of the clamp (which faced black side of the electrophoresis holder): sponge, western blotting filter paper (Thermo Scientific), gel, activated membrane,

western blotting filter paper and finally sponge. The gel tank was then filled with 1x transfer buffer and an ice pack placed into the tank along with the clamp containing the gel and membrane. This was run at 30 volts overnight.

The following day the membrane was then blocked with shaking for an hour in 5% milk solution in TBST at room temperature. The membrane was washed in TBST to remove any excess milk. The membrane was incubated with shaking in the primary antibody (either anti ubiquitin with a concentration of 1:5,000 or anti GST with a concentration of 1:5,000 or anti YFP with a concentration of 1:10000) for 1-3 hours at room temperature. Three washes of 5 minutes with TBST were then performed at room temperature. The membrane was then incubated with the secondary anti body 1:10,000 (rabbit for anti ubiquitin, rat for anti GST and rat for anti YFP) for 1 hour with shaking at room temperature. To wash away any unbound and non-specifically bound antibodies the membrane was washed 5 times for 5 minutes in TBST at room temperature. Then 2ml of ECL solution was washed over the membrane. After this the excess ECL solution was removed by dabbing the membrane on tissue and the membrane placed in between transparency film (Nice Day). Photographic film (Fujifilm, Tokyo, Japan) was then exposed to the membrane for differing time depending on the antibody. The film was then developed to visualize the protein bands.

2.14. Plant growth conditions

The wheat variety Avalon was grown in long day conditions, 16 hours at 24^0C and 8 hours at 24^0C. The *N. benthamiana* plants were grown in long day conditions. 16 hours at 24^0C and 8 hours at 24^0C.

2.15. Virus Induced Gene Silencing (VIGS)

To silence wheat the BSMV with the wheat gene fragment in *Agrobacterium* was first infiltrated into *N. benthamiana* to allow the virus to multiply. The *Agrobacterium* was grown in an overnight 10ml LB culture and spun down in the morning to get the bacterial pellet. The pellet was resuspended in 10mM $MgCl_2$ to an O.D. 600 of 1.5. To this mixture the same amount of μl to ml volume of acetosyringone (0.1mM) (Sigma Aldrich) was added and left to incubate at room temperature for 3 hours. An equal amount of α, β and modified γ BSMV were mixed together and then infiltrated into the 4-8th leaf of a 4 week old *N. benthamiana* plant. This was left to grow for a week. The infiltrated leaf was then ground up with water and rubbed onto a wheat leaf (2 weeks old) that had been previously sprinkled with carborundum powder (Fischer Scientific). All the wheat plant leaves were infiltrated and left to grow for 2 weeks to allow the silencing to take effect (successful silencing was seen in the PDS silenced plant).

2.16. Septoria infection

The plants to be infected were trimmed so that only the leaf to be infected was left. The remaining leaves were then stuck down flat onto black card, leaving ~5cm of leaf to be infected. The Septoria strain IPO323, K5090 (GFP strain) and K5097 (GFP strain) were spread onto YPD plates containing no antibiotics (IPO323) or YPD + hygromycin (K5090 and K5097, 25μg/ml) and left for 4 days to grow at 18^{0}C. A few streaks of the Septoria were taken from the plate and dissolved into 10ml of distilled water. To measure the concentration of the Septoria spores 10μl of Septoria mixture was pipetted onto a haemocytometer slide. Using an Axkiospop microscope at x20 magnification the spores were counted. The concentration was then adjusted to, on average, 4 spores 0.05*0.05mm square (1,000,000 spores per ml). 0.1% Tween 20 was added to the Septoria mixture.

Using a cotton bud the Septoria was infected onto the leaves by rubbing the mixture onto the leaves. A new cotton bud was used for each new silenced line of wheat. The tray under the plants was filled with water and the plants were covered with a lid to generate a high humidity for the Septoria to infect. The lids were removed after 4 days. The plants infected with IPO323 were left for 28 days with pictures taken daily and samples taken weekly to check for silencing. The final infected leaves were collected for the spore count, pycnidia count and for extraction of RNA to measure the amount of a fungal gene present. The plants infected with the GFP Septoria strains (K5090 and K5097) were grown and small sections of 0.5cm^2 were cut and mounted on a slide to be visualized on a SP5 confocal microscope.

2.17. Spore and pycnidia counts

The leaves were collected and suspended in a sealed box containing damp tissue to increase the humidity and cause the fungus to produce spores. This was left at 18^{0}C for 4 days. The pycnidia were counted over a 2cm length of each leaf and averaged for 5 leaves. 5 leaves were then submerged in 10ml of distilled water, vortexed for 2 minutes and left to stand for 3 hours. The leaves were vortexed again for 2 minutes and 10μl of the water loaded onto a haemocytometer slide. The spores were counted for 4 of the 0.2*0.2mm squares on the Akioskop light microscope at x20 magnification and an average taken from these 4 numbers for the spore count.

2.18. Transient expression in *Nicotiana benthamiana*

For transient assay in *N. benthamiana* the gene to be expressed was cloned into a vector containing an YFP N terminal fusion (pEarleyGate104) and transformed into *Agrobacterium*. A 10ml LB culture of this and P19 (RNAi silencing inhibitor) with the appropriate antibiotic (kanamycin) was shaken overnight at 28^{0}C. The culture was then centrifuged for 5 minutes at 5,000rpm, 4^{0}C. The supernatant

was then poured away leaving the bacterial pellet. The pellet was then resuspended in 10mM $MgCl_2$ to an O.D. 600 of 0.4 and 0.1mM of acetosyrine was added. The mixture was left for 1 hour at room temperature. A 50:50 mixture of the gene to be expressed and P19 was made and then infiltrated into *N. benthamiana* leaves using a 1ml syringe. The plants were watered and left for 8 days before a small section, 0.5cm^2, was used to visualize the YFP on a SP5 confocal microscope.

2.19. Confocal microscopy

A section of leaf 0.5cm^2 was mounted onto a slide (Fischer Scientific) in water and a 22x22mm cover slip (Menzel-Glaser, Waltham, USA) was tightly secured over with micropore tape. Either the YFP or GFP setting was used on the LASII software and the x60 OIL objective was selected. A small drop of oil was put onto the objective and the slide was loaded upside down with the coverslip on the side of the objective. Firstly the eyepiece was used to roughly focus the sample using a TLF-GFP light. Then using the software the image was focused fully before capturing it. During the focusing and scanning for an image the speed was set at 700, resolution at 512x512, 1 line average, pinhole airy 1 and bidirectional scanning. To capture a high resolution image the speed was set at 100, resolution at 1024x1024 5 line average and pinhole airy 1.

3. TaU4 is a negative regulator of wheat defence against Septoria

3.1. Introduction

Figure 1

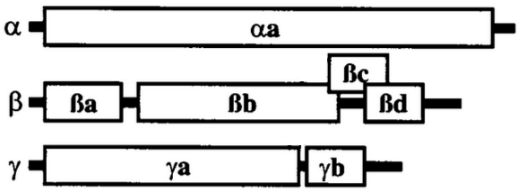


Figure 1: A schematic diagram of BSMV αRNA, βRNA and γRNA. αa gene encodes for a helicase. βa encodes for the coat protein. βb, βc and βd encode for proteins involved in viral movement. γa encodes for the RNA polymerase and γb encodes for a RNA binding protein involved in pathogenecity (Holzberg et al 2002). Figure modified from Holzberg et al 2002.

As generating wheat knockout mutants is difficult and time consuming due to the large hexaploid genome, wheat genes can be transiently silenced using VIGS technique. Holzberg et al (2002) were the first group to employ the BSMV virus for silencing barley genes. BSMV also naturally infects other monocot species such as wheat, oats and maize (McKinny and Greeley 1965) therefore can be used to silence genes in these species. The BSMV strain used for silencing contains three positive sense, single stranded RNA's designated α, β and γ (Dolja, 1979; Bragg and Jackson 2004) (Figure 1). Holzberg et al (2002) modified the BSMV virus to increase infectivity and therefore silencing (by deleting the coat gene from the βRNA) and to allow a fragment of a plant gene to be cloned into the γRNA (by modifiying the γb gene after the stop codon). BSMV vector has also been used to successfully silence wheat genes, for example *phytoene desaturase* (*PDS*), *magnesium-protoporphyrin chelatase complex subunit H* (*Ch1H*) and *β7 subunit of the 20S proteosome* (*20S-β7*). The silencing of each of these genes leads to a visible phenotype of photobleaching, chlorosis and necrosis respectively. To quantify the visible phenotype the level of the silenced gene mRNA was measured using reverse transcription PCR, showing no amplification with increasing numbers of cycles. The symptoms of BSMV infection upon wheat have been assessed and found to be less severe in wheat than in barley. Lessening the symptom severity of BSMV allows for a more accurate assessment of gene function in disease resistance studies (Scofield et al 2005).

Figure 2

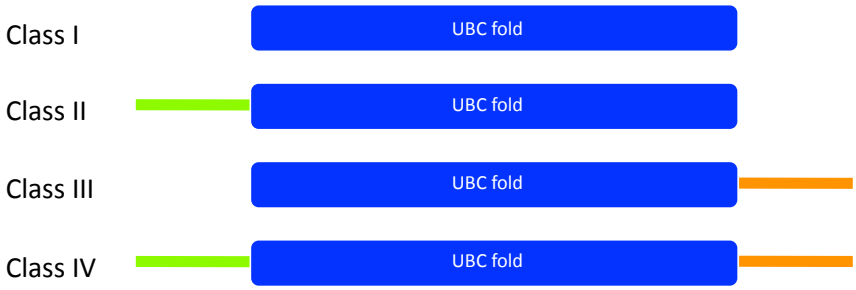


Figure 2: A schematic of the different classes of E2 ubiquitin conjugating enzyme. The UBC fold is indicated by a blue rectangle, a green line indicates the N terminal extension and an orange line indicates the C terminal extension. Figure modified from Wijk and Timmers 2010.

A previous study (Lee et al unpublished) found several different genes, coding for ubiquitination pathway enzymes, whose expression levels changed after Septoria infection. From these genes a putative E2 enzyme, designated *Triticum aestivum ubiquitin conjugating enzyme 4* (*TaU4*) was chosen for further investigation.

Few studies have looked at the effects of E2 enzymes in pathogen defence, this chapter aims to investigate a possible role one E2 enzyme has in the Septoria-wheat interaction using VIGS techniques.

3.2. TaU4 analysis

TaU4 is predicted to be an E2 enzyme based on the comparison of TaU4 protein sequence to other enzymes whose E2 function has already been defined (Figure 3). All E2 enzymes contain a conserved region of 150-200 amino acids, which is the ubiquitin conjugating catalytic (UBC) fold. Within this fold is the active site cysteine that binds to the ubiquitin (Burroughs et al 2007). The active site cysteine of the TaU4 is predicated to be at cysteine 88 based on the alignment of TaU4 to other known E2 enzymes, as shown in Figure 3. As shown in Figure 2 there are four classes of E2 enzymes, class I are E2 enzymes made up of just the UBC fold, class II have an N terminal extension, class III have a C terminal

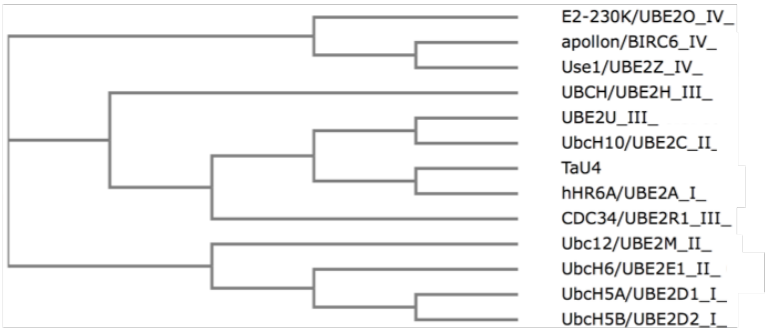
extension and class IV have both an N terminal and C terminal extension on the UBC fold (Wijk and Timmers 2010). A phylogenetic tree was generated using Clustaw Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/), in which the protein sequence of TaU4 was compared to human E2 enzymes that have previously been classified into each group. TaU4 appears most closely related to E2 enzymes from class I, II and III. It is a relatively small protein, with a coding sequence of 459 base pairs and a molecular weight of 17.4kDa. TaU4 protein sequence was then compared to E2 enzymes with known functions, including ones involved in defence against pathogens (FcUBC from shrimp (*Fenneropenaeus chinensis*) and OsUBC5b from rice (*Oryza sativa*)). Surrounding the active site cysteine (highlighted in black) there is a high degree of amino acid similarity between the different E2 enzymes, especially between the plant and defence E2 enzymes (Figure 3c). TaU4 is most closely related to the E2s from *A. thaliana*, which involved in the timing of flowering (AtUBC1 and AtUBC2) and regulation of the cell cycle (AtUBC19).

Figure 3:

(a)

```
E2-230K/UBE2O(IV)   1013 PAVPPHFCYLS--QCSGRLNPNLYD-NGKVCVSLLGTWIGK------GTERWTSKSSL-L 1062
apollon/BIRC6(IV)   4637 PSSPPLVNLETTGGHSVRFNPNLYN-DGKVCLSILNTWHG------RPEEKWNPQTSSFL 4689
UBCH/UBE2H(III)       62   PFKSPSIGFMN-----KIFHPNIDEASGTVCLDVIN-------------QTWTALYDL-T 102
UBE2U(III)            64   NYAPPVVKFIT-----IPFHPNVDPHTGQPCIDFLDNP-----------EKWNTNYTL-S 106
Ubc12/UBE2M(II)       87   PHDPPKVKCET-----MVYHPNIDL-EGNVCLNILR-------------EDWKPVLTI-N 126
CDC34/UBE2R1(III)     69   PYSPPAFRFLT-----KMWHPNIYE-TGDVCISILHPPVDDPQSGELPSERWNPTQNV-R 121
Use1/UBE2Z(IV)       159   PIHPPRVKLMTTGNNTVRFNPNFYR-NGKVCLSILGT---------WTGPAWSPAQSI-S 207
UbcH10/UBE2C(II)      90   PYNAPTVKFLT-----PCYHPNVDT-QGNICLDILK-------------EKWSALYDV-R 129
TaU4                  64   PNKPPTVRFIS-----RMFHPNIYA-DGSICLDILQ-------------NQWSPIYDV-A 103
hHR6A/UBE2A(I)        64   PNKPPTVRFVS-----KMFHPNVYA-DGSICLDILQ-------------NRWSPTYDV-S 103
UbcH6/UBE2E1(II)     107   PFKPPKVTFRT-----RIYHCNINS-QGVICLDILK-------------DNWSPALTI-S 146
UbcH5A/UBE2D1(I)      61   PFKPPKIAFTT-----KIYHPNINS-NGSICLDILR-------------SQWSPALTV-S 100
UbcH5B/UBE2D2(I)      61   PFKPPKVAFTT-----RIYHPNINS-NGSICLDILR-------------SQWSPALTI-S 100
```

(b)



(c)

```
AtUBC1       66 KPPTVRFVSRMFPPNIYA-DGSICLDILQNQWSPIYDVAAILTSIQSLLCDPNPNSPANSE 125
AtUBC2       66 KPPTVRFVSRMFPPNIYA-DGSICLDILQNQWSPIYDVAAILTSIQSLLCDPNPNSPANSE 125
TaU4         66 KPPTVRFISRMFPPNIYA-DGSICLDILQNQWSPIYDVAAILTSIQSLLCDPNPNSPANSE 125
AtUBC19      97 KSPKVKFETCCFPPNVDL-YGNICLDILQDKWSSAYDVRTILLSIQSLLGEPNISSPLNNQ 157
OsUBC5b      63 KPPKVALKTKVFPPNINS-NGSICLDILKEQWSPALTISKVLLSICSLLTDPNPDDPLVPE 122
OsUBC        63 NRPKVAFKTKVFPPNINS-NGSICLDILKEQWSPALTVSKVLLSICSLLTVPNPDDPLVPE 122
FcUBC        64 QPPQIKFKTPIYPMNVGP-YGDICLDILDKNWSPALSISKVLLVICVLMTDPNPDDPLRFN 123
AtCOP10      97 KPPKLVFKTRIYPCNVDT-AGDLSVNILRDSWSPALTITKVLQAIRSIFLKPEPYSPALPV 157
LeUBC1       66 EPPKMKFATKVWPPNISSQSGAICLDILKDQWSPALTLKTALLSIQALLSAPEPDDPQDAV 125
```

(d)

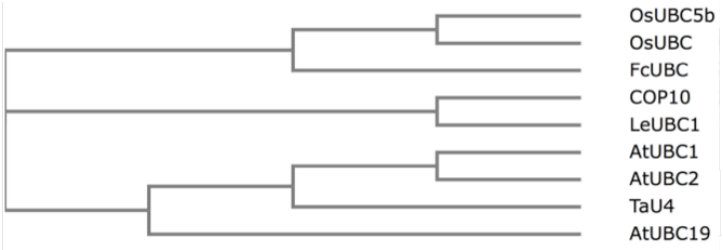


Figure 3: TaU4 protein sequence alignment to other known E2 enzymes protein sequences using Clustlaw Omega. (a) The protein sequence of TaU4 was aligned to human E2 enzymes protein sequences that have already been assigned a class (Class I-IV, figure 2) based on their structure. **(b)** A phylogenetic tree was generated using Clustlaw omega comparing the protein sequences of the human E2's with TaU4 to show closely related E2 enzymes. **(c)** TaU4 protein sequence was aligned to E2 enzymes whose E2 enzyme function has been defined, including E2 enzymes involved in defence. **(d)** A phylogenetic tree was generated to compare the protein sequences of known E2 enzymes to TaU4. At = *Arabidopsis thaliana*, Ta = *Triticum aestivum*, Os = *Oryza sativa*, Le = *Lycopersicon esculentum* and Fc = *Fenneropenaeus chinensis*. Accession numbers for the sequences used in this figure are: E2-230K/UBE2O = NM_022066.3, apollon/BIRC6 = NM_016252.3, UBCH/UBE2H = BC006277.2, UBE2U = BC029895.1, Ubc12/UBE2M = NM_003969.3, CDC34/UBE2R1 = NM_152489.1, Use1/UBE2Z = NM_004359.1, UbcH10/UBE2C = BC007656.2, hHR6A/UBE2A = DQ068065.1, UbcH6/UBE2E1 = BC009139.1, UbcH5A/UBE2D1 = BC015997.1, UbcH5B/UBE2D2 = BC033349.1, AtUBC1 = , AtUBC2 = , AtUBC19 =

3.3. TaU4 expression levels in healthy and infected tissue

The involvement of *TaU4* in wheat defense against Septoria is suggested by the study of *TaU4* gene expression level changes during Septoria infection. In the present study healthy and Septoria infected wheat leaves were collected every two days after infection. RNA was extracted from this leaf tissue and used to synthesis cDNA for use in real time PCR (Figure 4). The levels of *TaU4* expression are low in comparison to the constitutively expressed housekeeping gene (*18S RNA*) that was used to normalize *TaU4* gene expression. *TaU4* gene expression levels fluctuate in the healthy wheat leaf samples, with a slight increase in gene expression every 6-8 days for 4 days before decreasing again. The gene expression increases at 12-14 days, then decreases, back down similar levels to that of 10dpi (days post infection) and before, at 16dpi. The same pattern occurs for the infected tissue however at the onset of visible disease symptoms (12-14dpi) the expression level is lower in the infected tissue than the healthy tissue. Throughout the rest of the infection *TaU4* mRNA levels are higher in the infected

tissue than the healthy tissue. Septoria infection appears to also induce a more consistent, but sometimes lower, expression of *TaU4*, with less expression fluctuation in the infected tissue compared to the healthy tissue (Figure 4).

Figure 4:

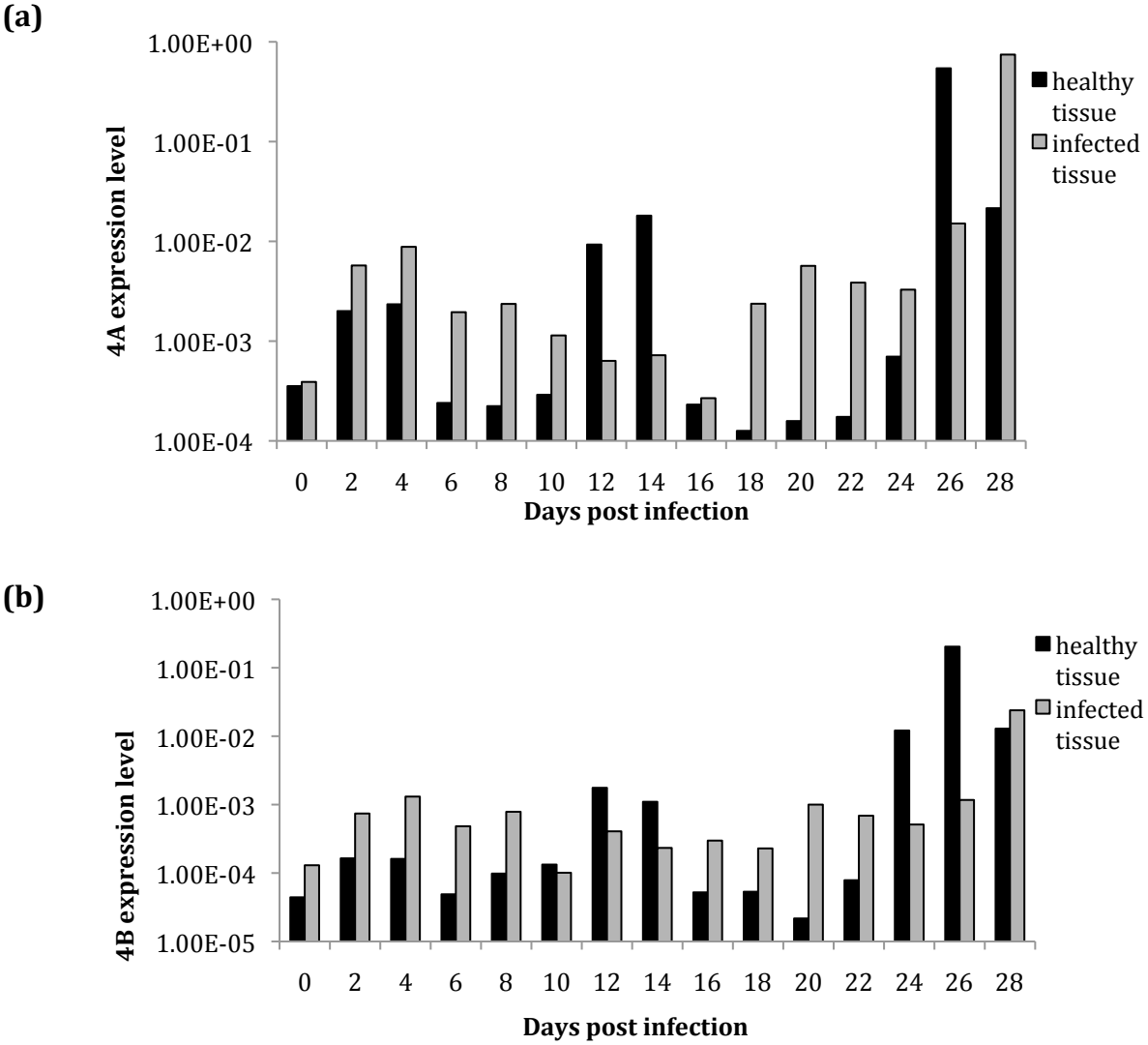


Figure 4: Expression levels of *TaU4* in infected and healthy wheat measured for 28 days after infection. Samples were collected every two days after infection for RNA extraction and cDNA synthesis. Expression levels were then measured with real time PCR using two independent specific primers for *TaU4*, **(a)** 4A and **(b)** 4B. Expression levels were normalized against the expression of *18S rRNA* of wheat. The real time PCR was only repeated once due to time constraints. Two leaves were collected each time but this again was only repeated once.

3.4. VIGS with PDS positive control

Reverse genetics techniques, such as VIGS used for transient gene silencing, are useful techniques for defining gene product functions. A positive control for VIGS is the silencing of *PDS* wheat gene. *PDS* is involved in the biosynthesis of carotenoids, which (when silenced) allows chlorophyll damage to occur resulting in a bleached leaf phenotype that is easily visible (Scolnik and Bartley 1993). The phenotype becomes visible after approximately 14 days of silencing, as seen in Figure 5a. As well as the visible phenotype, the levels of *PDS* mRNA can be measured after silencing using real time PCR (Figure 5b), only one repeat was performed due to time constraints. After silencing there was a reduction of 82% in the levels of *PDS* mRNA compared to non-silenced wheat (Figure 5b).

Figure 5:

(a)

(b)

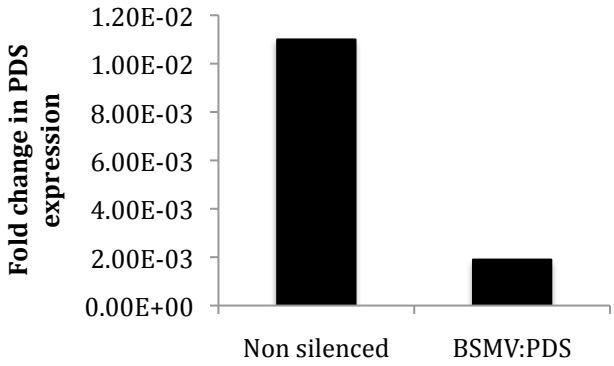


Figure 5: The use of PDS silenced wheat as a positive control for silencing. **(a)** White light pictures of *PDS* silenced wheat (2 weeks after silencing treatment) showing a visible white phenotype compared to non-silenced wheat. **(b)** RNA was extracted and cDNA generated from non silenced and *PDS* silenced wheat. This was then used in real time PCR to measure the expression levels of *PDS*. Expression levels were normalized against the expression of *18S rRNA* of wheat. *BSMV:PDS* silenced is 82% lower in expression compared to non silenced wheat.

3.5. *BSMV:TaU4A* and *BSMV:TaU4B* silencing

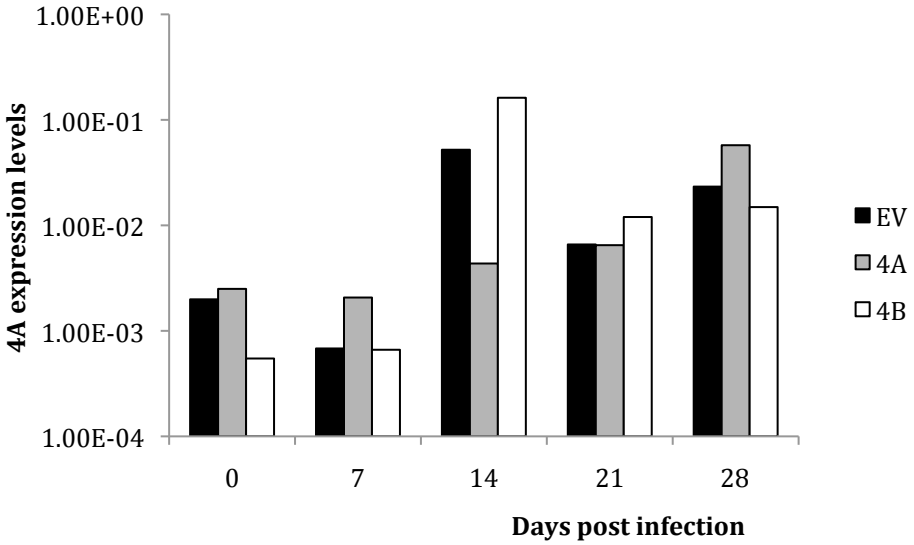
To study TaU4 effects in Septoria-wheat interaction VIGS was used to silence *TaU4* in wheat before infecting the wheat leaves with Septoria fungus. Two independent specific fragments of 200 nucleotides were used for *TaU4* silencing, *BSMV:TaU4A* and *BSMV:TaU4B* (appendix). Using two independent specific fragments helps to avoid any potential off target silencing and because different fragments can have different levels of silencing (Thomas et al 2000; Luo and Chang 2004; Birmingham et al 2006).

Samples of the silenced wheat were taken every 7 days post Septoria infection for RNA extraction and cDNA synthesis; the levels of *TaU4* expression were then measured from *BSMV:00*, *BSMV:TaU4A* and *BSMV:TaU4B* leaf tissue to check for silencing (Figure 6). Two independent specific primers sets were used in the real time PCR to avoid any amplification of the *TaU4* fragment encoded for by the BSMV virus that would lead to supposed higher levels of plant expressed *TaU4* mRNA. For the real time the primer sets were first tested using serial cDNA dilutions, this indicated that 4A primers were more accurate than 4B, however both were suitable for real time PCR. Only one repeat of the real time PCR was performed due to time constraints.

Figure 6 shows silencing data for 28 days of infection for *BSMV:00*, *BSMV:TaU4A* and *BSMV:TaU4B* silenced wheat measured with 4A (Figure 6a) and 4B primers (Figure 6b). As can be seen in Figure 5 the silencing of TaU4 in both *BSMV:TaU4A* and *BSMV:TaU4B* is not consistent throughout the experiment when measuring with both primer sets. Silencing appears more complete during the start of the experiment (0 and 7dpi) compared to the latter days (21 and 28dpi). Both the primer sets show a similar pattern in the expression levels of TaU4 throughout the silencing experiment in comparison to the *BSMV:00* control.

Figure 6:

(a)



(b)

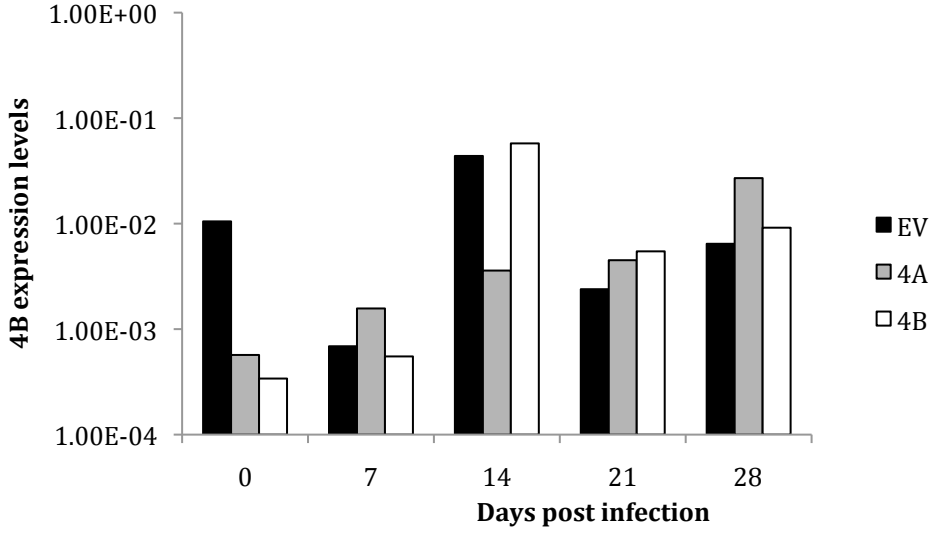


Figure 6: Expression levels of *TaU4* in *BSMV:00*, *BSMV:TaU4A* and *BSMV:TaU4B* silenced wheat. RNA extracted from *BSMV:00*, *BSMV:TaU4A* fragment and *BSMV:TaU4B* fragment silenced wheat every 7 days after the wheat leaves were infected with Septoria (2 weeks after silencing treatment). cDNA was synthesised from this RNA for real time PCR. The expression level of *TaU4* was then measured with **(a)** 4A primers and **(b)** 4B primers**.** Expression levels were normalized against the expression of *18S rRNA* of wheat. EV = *BSMV:00*, 4A = *BSMV:TaU4A* and 4B = *BSMV:TaU4B*

3.6. Septoria infection upon BSMV:*TaU4* silenced wheat

Figure 7 shows the symptoms of Septoria infection over 28 days in wheat leaves silenced with *BSMV:00, BSMV:TaU4A and BSMV:TaU4B* fragments. The visible Septoria infection symptoms start to develop at 14 days post infection, with *BSMV:00* wheat showing the most severe disease symptoms at this time point. There is some necrosis at the top of the *BSMV:00* leaf in comparison to *BSMV:TaU4A* and *BSMV:TaU4B* silenced leaves that show little to no necrosis, with the leaves mostly retaining a green colouring. The difference between the *BSMV:00, BSMV:TaU4A* and *BSMV:TaU4B* silenced wheat Septoria disease symptoms are most noticeable at 21dpi, with *BSMV:00* showing full leaf necrosis whereas *BSMV:TaU4A* and *BSMV:TaU4B* both still retain some green colouring. *BSMV:TaU4A* appears to be more resistant to Septoria disease symptoms than *BSMV:TaU4B*, which could be due to a difference in silencing efficiencies between the two fragments. By 28dpi all three of the silenced lines show full necrosis as Septoria begins to produce spores (Figure 7).

Figure 7:

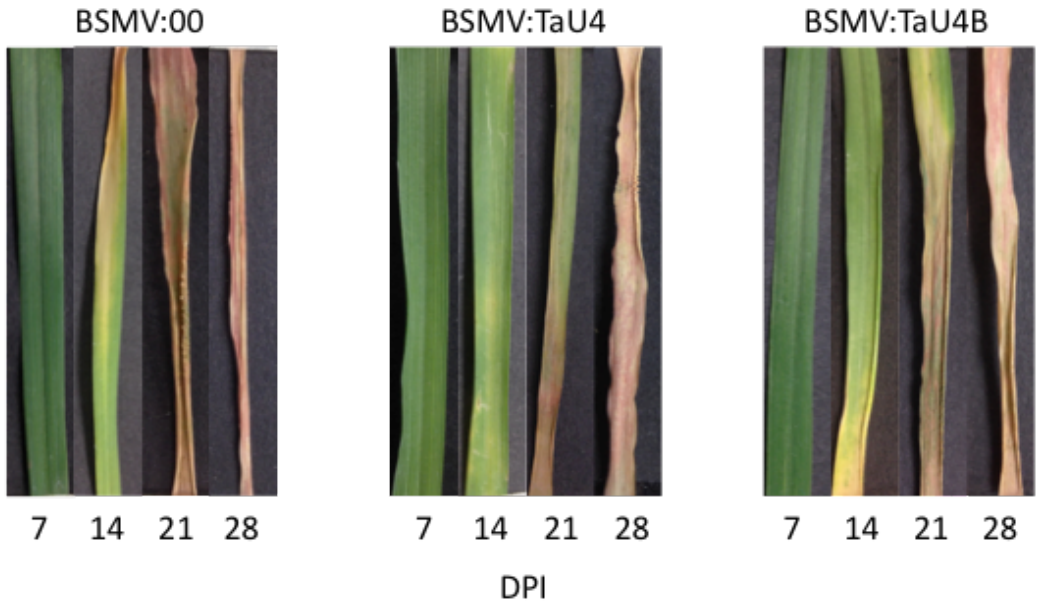


Figure 7: *BSMV:00*, *BSMV:TaU4A* and *BSMV:TaU4B* silenced wheat after infection with Septoria fungus. White light pictures were taken every 7 days up to 28 days after infection to follow the disease symptoms of the Septoria and differences between the symptoms for the different silenced wheat leaves. DPI = days post infection. DPI = days post infection.

3.7. Pycnidia and spore counts for *BSMV:TaU4A, BSMV:TaU4B* and *BSMV:00* silenced wheat after 28 days of Septoria infection

After 28 days of Septoria infection the infected leaves were collected and put into high humidity for 3 days to allow the pycnidia to form and spore production for counting.

Figure 8a shows Septoria pycnidia and spore counts for wheat silenced with *BSMV:00, BSMV:TaU4A* and *BSMV:TaU4B* fragments. There is a significant difference ($p<0.05$) in the pycnidia counts between *BSMV:00* and *BSMV:TaU4A* and *BSMV:00* and *BSMV:TaU4B* silenced wheat leaves when measured with a student t test. This shows that the silencing treatment of *TaU4* causes a reduction in the amount of pycnidia produced by Septoria, which implies there is less Septoria to produce pycnidia or that pycnidia production is negatively effected. The wheat silenced with *BSMV:TaU4A* fragment has a greater effect on the Septoria pycnidia production than that of the wheat silenced with *BSMV:TaU4B*, which could be related to the different fragments ability to silence *TaU4* (Thomas et al 2000; Luo and Change 2004; Birmingham et al 2006). The number of pycnidia on both *BSMV:00* and *BSMV:TaU4B* silenced wheat is variable over the different wheat leaves tested, as shown by the error bars (1 standard deviation), however there are still significant differences between the data sets.

The spore count (Figure 8b) correlates with the pycnidia count as *BSMV:00* has a significantly higher number of spores than *BSMV:TaU4A* ($p<0.05$) and on average a higher number than *BSMV:TaU4B* when compared using a student t test. This is to be expected as the spores are produced within the pcynidia (Eyal 1999; Orton et al 2011). The spore count for Septoria infecting the *BSMV:00* silenced wheat leaves was much more variable than for *BSMV:TaU4A* or *BSMV:TaU4B* silenced wheat leaves. This is shown by the error bar (1 standard deviation of the mean), which appears to overlap with both *BSMV:TaU4A* and *BSMV:TaU4B* error bars, however the difference is statistically significant under a student t test (Figure 7b). Together the pycnidia count and spore count show that there is more Septoria infecting the *BSMV:00* silenced wheat as opposed to the wheat silenced

with *BSMV:TaU4A* or *BSMV:TaU4B* fragments or that silencing of *TaU4* causes a reduction in Septoria fungus ability to reproduce asexually.

Figure 8:

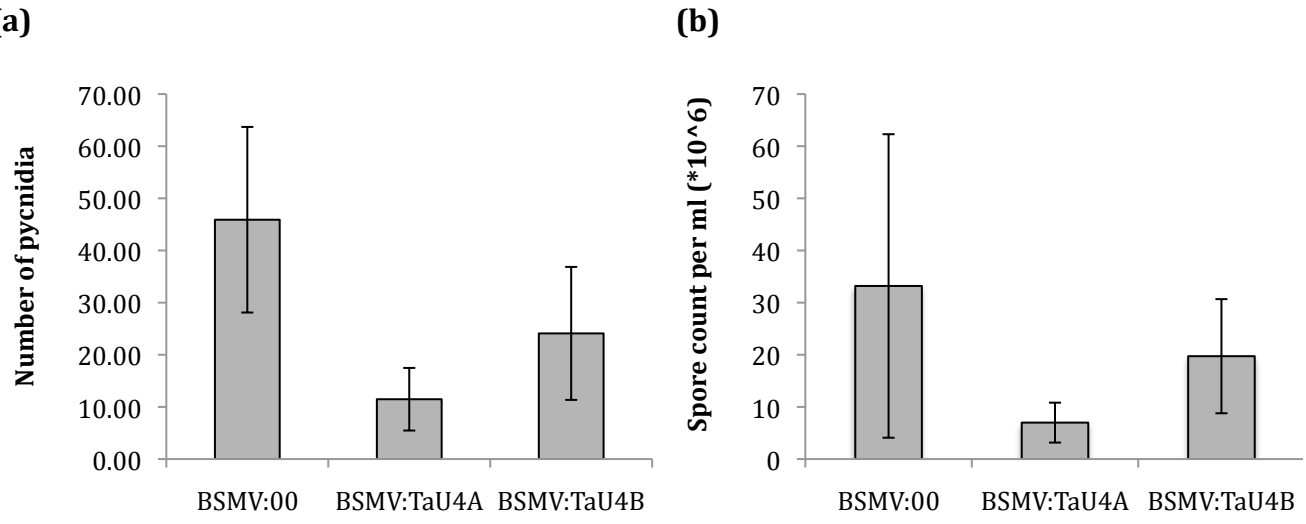


Figure 8: Pycnidia and spore counts for Septoria infected *BSMV:00*, *BSMV:TaU4A* and *BSMV:TaU4B* silenced wheat. (a) The pycnidia were counted over 2cm length of the leaf. The graph represents 1 technical repeat and 5 biological repeats. **(b)** The spores were measured using a haemocytometer slide and averaged across the 4 boxes of the slide. This was then used to work out the spores per ml (*10^6). This graph represents 3 technical repeats and 15 biological repeats. Error bars represent 1 standard deviation of the mean.

3.8. Measuring Septoria 60S L13 gene expression levels

Measuring Septoria ribosomal gene expression levels with real time PCR can be used to determine the amount of Septoria infection in wheat leaves. The primers for four different Septoria ribosomal proteins (two from the 40S subunit and two from the 60S subunit) were tested for their use in real time PCR. Out of the four only *60S L13* was found suitable for real time PCR after cDNA serial dilution analysis. Infected and healthy wheat leaf samples were collected every two days after infection over a time course of 28 days and the RNA extracted for cDNA synthesis. To show that there is an increase in Septoria after the infection the levels of Septoria *60S L13* gene expression were measured using real time PCR as shown in Figure 9. Only one set of leaf samples were collected and only one

repeat of this real time was performed due to time constraints. The levels of expression are very low at first and increase steadily up until 14-16dpi, where they increase rapidly. This rapid increase in expression of *60S L13* correlates with the switch between biotrophic and necrotrophic growth of Septoria. The expression of *60S L13* after 16dpi then maintains itself steadily until 28dpi were there is a large increase of over 100% in *60S L13* expression as the Septoria prepares for sporulation.

Figure 9:

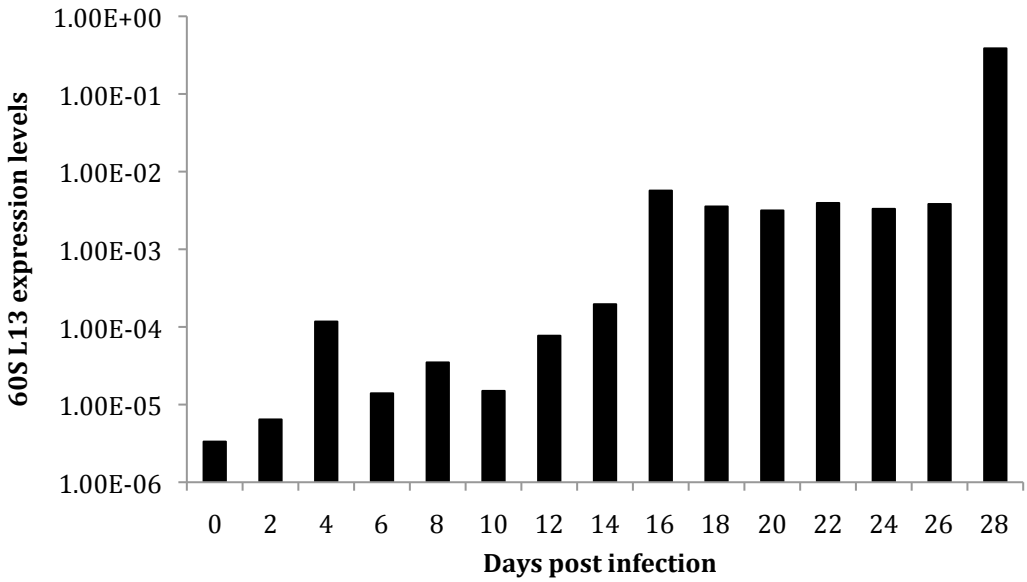


Figure 9: Real time PCR was used to measure the expression levels of Septoria 60S L13 ribosomal protein in infected wheat over 28 days of infection. Samples were collected every 2 days from the start of the infection until 28 days after infection for RNA extraction and cDNA synthesis. Expression levels were normalized against the expression of 18S rRNA of wheat.

4. TaU4 is an E2 ubiquitin conjugating enzyme

4.1. Introduction

TaU4 has been found to have a function in Septoria-wheat interaction and is suspected to be an E2 enzyme, however the E2 activity of TaU4 has yet to be proven. The ability of E2 enzymes to ubiquitinate a target substrate (either directly or indirectly via an E3 enzyme) relies on the ubiquitin becoming bound to a cysteine residue on the E2 enzyme via a thioester bond. This occurs only after the ubiquitin has been activated, with ATP, by an E1 enzyme.

The aim of this chapter is to show that TaU4 is a functioning E2 enzyme. Previous studies of E2 enzymes have used ubiquitin charging assays to prove E2 enzyme functioning capabilities (Goebl et al 1988; Number et al 1996; Johnson and Blobel 1997). An E2 ubiquitin charging assay was therefore used to investigate whether ubiquitin can bind to TaU4 through a thioester bond and that it is dependent on other necessary components of the ubiquitin pathway (E1 enzyme, ATP, ubiquitin) and the active site cysteine.

4.2. TaU4 and TaU4^{C88S} mutant cloning

The full length TaU4 protein coding sequence was cloned from extracted wheat leaf cDNA into the destination vector pDEST15 (with an N terminal GST tag) and transformed into BL21 *E. coli*. To prove that the active site cysteine is needed for TaU4 to bind to ubiquitin a mutant *TaU4* gene was generated with cysteine substituted for the structurally similar but chemically different amino acid, serine. Site directed mutagenesis was used to change a thymine nucleotide base to an adenine nucleotide base, changing the DNA sequence from tgc to agc therefore substituting the amino acid cysteine to serine. To ensure that the site directed mutagenesis was successful the PCR product was incubated with the restriction enzyme HindIII that recognizes the restriction site 5'-a|agctt-3', which is present once in the mutant *TaU4*^{C88S} but not in the *TaU4* wildtype DNA (Figure

10). This is shown in the DNA gel electrophoresis picture in Figure 11 as two bands of sizes 198 and 261 compared to the one band of size 459 in the *TaU4* wildtype.

Figure 10:

TaU4						
a g c a t a t g c t t a g a t						
86	S	I	C	L	D	90
TaU4^{C88S}						
a g c a t a a g c t t a g a t						
86	S	I	S	L	D	90

Figure 10: The coding sequence and protein sequence of the active site of *TaU4* wild type and the *TaU4^{C88S}*. The active site cysteine (amino acid position 88) and mutated serine are highlighted in red and the recognition site of the restriction enzyme HINDIII is highlighted in blue.

Figure 11:

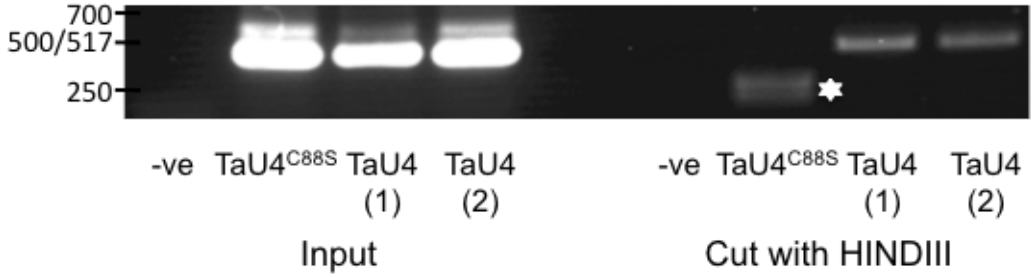


Figure 11: DNA gel electrophoresis picture of wild type *TaU4* and mutated active site *TaU4^{C88S}* cut with HINDIII restriction enzyme. Figure 10 shows that HINDIII only recognises mutated *TaU4^{C88S}* DNA sequence. PCR amplification of *TaU4^{C88S}* and two independent clones of *TaU4* from D-TOPO using *TaU4* specific primers were used as shown in the input. This was then digested with HINDIII (right hand side). The negative control uses water as a template. Undigested *TaU4^{C88S}* and *TaU4* = 459bp and digested *TaU4^{C88S}* = 198 and 261 as indicated with the star.

4.3. TaU4 and TaU4^{C88S} mutant protein expression

GST:TaU4 and *GST:TaU4^{C88S}* (pDEST15 vector) were transformed into BL21 for protein expression use. The protein expression system is activated in the presence of IPTG (a non-hydrolysable lactose analogue). When IPTG is present it causes the conformational change of the Lac operon repressor, *LacI*, so that it can no longer bind to the DNA and repress expression of the Lac operon genes (*LacY permease*, *LacZ β-galactosidase* and *LacA thiogalactoside transacetylase*). The

protein to be expressed has a promoter from the Lac operon, therefore is also induced by IPTG. After the addition of IPTG the protein of interest's cellular concentration can increase by up to x1,000 times (Beckwith 1967, Bell and Lewis 2000).

Expression of Lac operon genes is not completely switched off in the absence of an inducer and so there is a low level of expression of the protein. An extra level of control in the pDEST15 vector used to express *TaU4* has been introduced, where *TaU4* has a T7 bacteriophage promoter not recognized by *E. coli* RNA polymerase. The viral T7 bacteriophage RNA polymerase is encoded for in the BL21 *E. coli* strain genome and is also under the control of LacI therefore induced by IPTG. Even with this extra level of control the system can still be leaky. This can be seen in Figure 12 that shows a western blot (anti-GST) of the expression of *TaU4* and *TaU4*^{C88S}, which are both expressed highly before the addition of IPTG (0 Hour). The protein is expressed mostly in the soluble fraction (S) rather than the insoluble fraction (I). Introducing IPTG increases the expression in the insoluble fraction and the breakdown (as seen by the smaller bands) for both proteins. With this in mind, the 1 hour collected fractions were used for the purification as it represents the best balance between high protein concentration in the soluble fraction and low protein breakdown (Figure 12).

Figure 12:

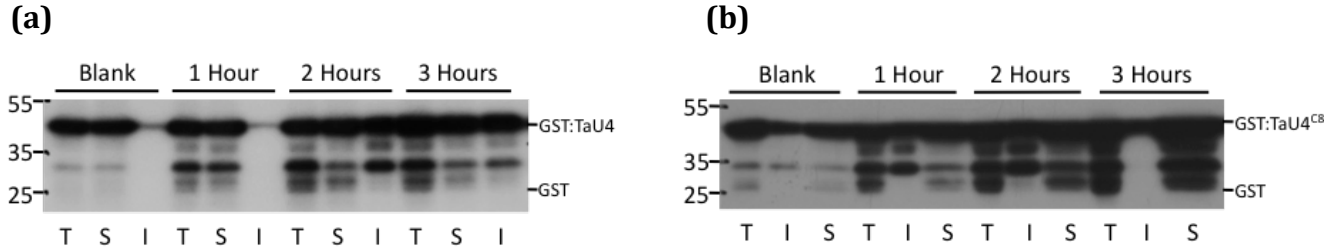


Figure 12: A western blot of TaU4 and TaU4^{C88S} protein expression visualized with anti-GST. Protein expression was induced by 1M IPTG and samples were taken before induction (blank) and 1 hour, 2 hours and 3 hours after induction with IPTG. Breakdown of proteins can be seen as the bands of size between TaU4 and GST and TaU4^{C88S} and GST. **(a)** *TaU4* expression. **(b)** *TaU4*^{C88S} mutant expression protein. T = total, S = soluble and I = insoluble. GST = 26kDa, GST:TaU4 = 43.4kDa and GST:TaU4^{C88S} = 43.4kDa.

4.4. TaU4 and TaU4^{C88S} mutant protein purification

Both TaU4 and TaU4^{C88S} protein were expressed with an N terminal GST tag (pDEST15), which was used for purification of the proteins using a GSTrap 4B purification column. Figure 13 shows a western blot (anti-GST) of the purified fractions of TaU4 and TaU4^{C88S} proteins. After the purification it was essential to remove the glutathione remaining in the buffer as this can interfere with the ubiquitin charging assay. As well as removing the glutathione, this process can be used to change the buffer and concentrate the protein. The buffer was changed from the extraction buffer to the ubiquitin charging assay buffer (minus the ATP). This was done for the fractions containing protein with the least breakdown of the GST from the protein (Figure 13) (fractions 5-10 for TaU4 and fractions 1 and 6-10 for TaU4^{C88S}).

Figure 13:

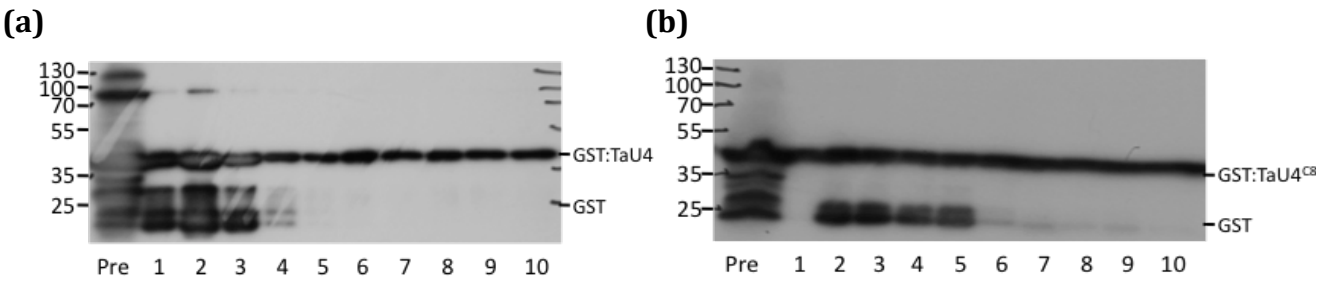


Figure 13: Western blot of the purified fractions of GST:TaU4 and GST:TaU4^{C88S} visualized with anti-GST. A pre purification sample is collected and 10 fractions of the purified proteins with GST tag are collected during column purification with GSTrap 4B column. GST breakdown from TaU4 and TaU4^{C88S} can be seen at around 26kDa. **(a)** The purified protein fractions of GST:TaU4. **(b)** The purified protein fractions if GST:TaU4^{C88S}. GST = 26kDa, GST:TaU4 = 43.4kDa and GST:TaU4^{C88S} = 43.4kDa.

4.5. E2 ubiquitin charging assays

For a ubiquitin charging assay the following components are essential, E1 enzyme, ubiquitin, ATP and E2 enzyme. Optimal conditions for the assay were found to be 30^{0}C for 5 minutes. The controls used in this assay include removing the E1 enzyme (-E1), removing the ATP (-ATP), removing the ubiquitin (-Ubq), TaU^{C88S} (as a negative control for the active site cysteine) and a known functioning E2 enzyme, UBCH5b (as a positive control). After the assay a reducing agent (DTT) was added to half of each assay sample. Under these reducing conditions the thioester bond between ubiquitin and the E2 enzyme can be broken (Figure 14). This can be used to demonstrate the binding of ubiquitin to TaU4 is through a thioester bond and not via an isopeptide bond such as the bond between ubiquitin and the target substrate.

Figure 14:

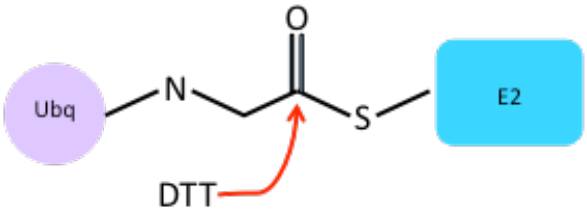


Figure 14: Schematic diagram of a thioester bond between an E2 enzyme and ubiquitin and the site of DTT reduction. This shows a thioester bond binding ubiquitin to active site cysteine of an E2 enzyme, which can be broken by reducing agent e.g. DTT. Ubiquitin is represented by the purple circle (Ubq) and the E2 enzyme by the blue rectangle.

Figure 15 shows the western blot for the E2 ubiquitin charging assay with anti-GST antibody. GST:TaU4 without His:ubiquitin is 43kDa, and can be seen in both the non-reduced and reduced blots in all the lanes containing TaU4. TaU4-ubiquitin (approximately 53kDa) can be seen in lane 1 (all) on the non-reduced blot but no TaU4^{C88S}-ubiquitin can be seen (lane 6), showing ubiquitin binds to the suspected active site at cysteine 88. The positive control is not visible in this blot as anti-GST antibody was used and the commercial UBCH5b E2 enzyme has a His-tag therefore should not be detected by anti-GST. When a component of the pathway is removed (E1 enzyme, ATP or ubiquitin) ubiquitin does not become bound to TaU4 as seen by the lack of band at approximately 53kDa in lanes 2, 3 and 4. After DTT is added the ubiquitin bound to TaU4 (lane 1) is no longer

present, showing that the ubiquitin is bound through a thioester bond. In both the reduced and non reduced conditions there is less TaU4^{C88S}, which could explain the lack of band at 53kDa. The mutated TaU4^{C88S} was much harder to purify to a high concentration compared to wildtype TaU4 protein but due to time constraints optimising the expression and purification of TaU4^{C88S} was not performed. GST breakdown from GST:TaU4 can also be seen on the reduced blot (Figure 15)

Figure 15:

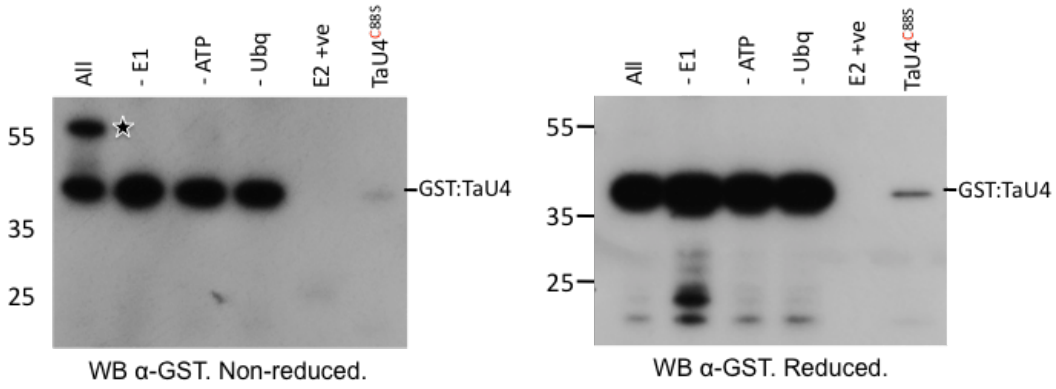


Figure 15: Western blot of E2 ubiquitin charging assay probed with anti-GST. The western blot shows a thioester reaction containing all the components necessary for the formation of an E2-ubiquitin intermediate. The negative controls are the subsequent removal of the E1, ATP, Ubiquitin (Ubq) and the use of the active site cysteine mutant TaU4^{C88S}. The positive control is the human E2 enzyme UBCH5b (His tagged). DTT was used as a reducing agent for the reduced assay (right hand side). GST breakdown from TaU4 can be seen as the bands below 43.4kDa.
(TaU4 = 17.37kDa, GST = 26kDa, ubiquitin = 9.5kDa, GST:TaU4 = 43.4kDa and UBCH5b = 18kDa).

To show that ubiquitin is binding to TaU4, the E2 ubiquitin charging assay was repeated and visualized through western blotting with anti-ubiquitin, Figure 16. In both lane 1 (all) and lane 5 (E2 +ve) there is a series of ubiquitinated TaU4 and UBCH5b in non reducing conditions (>53kDa and >26.5kDa respectively). After the addition of DTT there is less ubiquitinated TaU4 and UbcH5b, showing again that the ubiquitin is linked through a thioester bond. The remaining TaU4-ubiquitin and UBCH5b-ubiquitin in the reduced blot may be due to a low concentration of DTT or an insufficient incubation period with DTT, therefore leaving some E2 enzymes still bound to ubiquitin through a thioester bond. It is

also possible that the ubiquitin is linked instead through an isopeptide bond to the E2 enzymes. As with the anti-GST blot, the lanes lacking E1 enzyme, ATP, ubiquitin and the active site mutation (TaU4^{C88S}) have no TaU4-ubiquitin, again showing that ubiquitination of TaU4 needs an E1 enzyme, ATP, ubiquitin, an active site cysteine and a non-reducing environment. The ubiquitin antibody also appears to bind to the GST tag on TaU4 as well as the ubiquitin (shown at the bottom of the blot at approximately 10kDa). The breakdown of GST from both TaU4 and TaU4^{C88S} can be seen in both the non reduced and reduced blots of bands below 43.4kDa.

Figure 16:

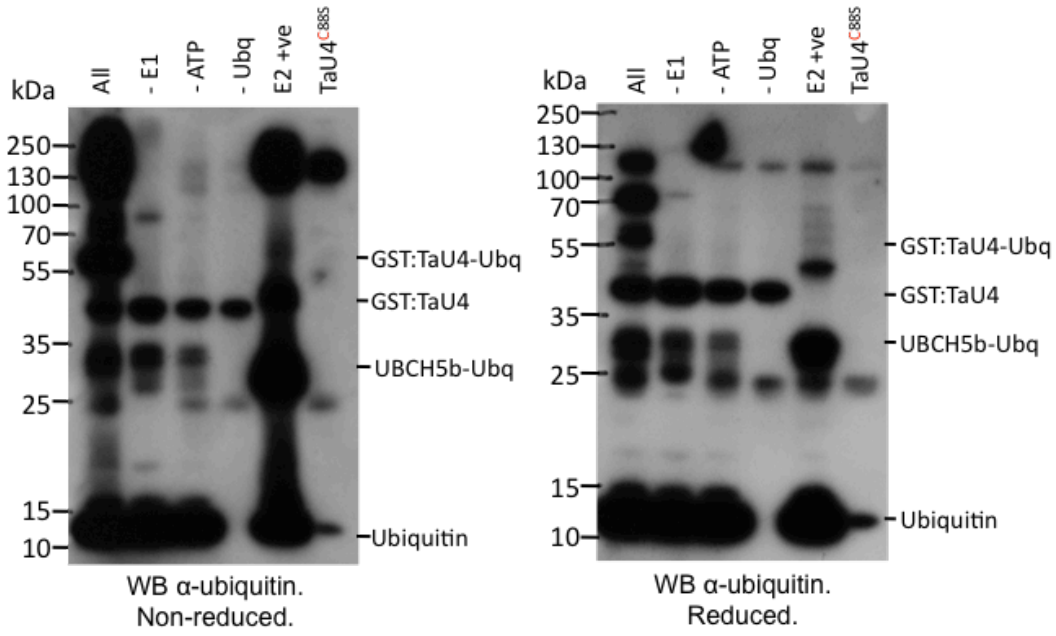


Figure 16: Western blot of E2 ubiquitin charging assay probed with anti-ubiquitin. The western blot shows a thioester reaction containing all the components necessary for the formation of an E2-ubiquitin intermediate. The negative controls are the subsequent removal of the E1, ATP, Ubiquitin (Ubq) and the use of the active site cysteine mutant TaU4^{C88S}. The positive control is the human E2 enzyme UBCH5b (His tagged). DTT was used as a reducing agent for the reduced blot (right hand side).
(TaU4 = 17.37kDa, GST = 26kDa, ubiquitin = 9.5kDa, GST:TaU4 = 43.4kDa and UBCH5b = 18kDa).

These western blots together show that TaU4 can be ubiquitinated via a thioester bond if an E1 enzyme, ubiquitin and ATP are present and that this is dependent on having a cysteine at the active site. This therefore proves that TaU4 is an E2 ubiquitin conjugating enzyme.

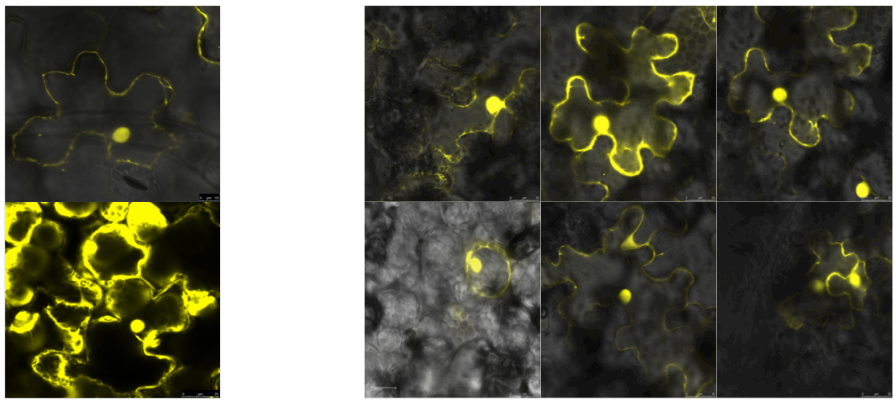
4.6. YFP:TaU4 localization studies in *N. benthamiana*

Working out the cellular localization can point to potential targets for TaU4 and interacting E3 enzymes. Agrobacterium transformed with a vector (pEarleyGate104) that expresses *TaU4* was infiltrated into *N. benthamaina*. Transient assays allow a fast and simple method for protein localization studies. pEarleryGate104 vector has an N terminal YFP tag before the att sites for cloning in the gene of interest. This can then be visualized using confocal microscopy (Voinnet et al 2003).

A YFP control, of *pEarleryGate104* without an insert, was used as a comparison to *YFP:TaU4*. As YFP is a small protein (27kDa) it can diffuse throughout the cytoplasm and into the nucleus as seen in Figure 17a The YFP:TaU4 protein is slightly larger, at 44kDa size. YFP:TaU4 is located in both the nucleus and the cytoplasm, similar to the localization of YFP (Figure 17b). To prove that the cellular localization seen in Figure 17b is due to YFP:TaU4 protein and not YFP alone the total protein was extracted from infiltrated *N. benthamiana* leaves. Figure 17c shows the western blot of the total extracted proteins that were separated based on size by a SDS PAGE gel and then visualized using anti-YFP antibody. Lane 1 contains the protein extracted from leaves infiltrated with *pEarleyGate104* vector only that shows a band at 27kDa, for YFP protein. The band then shifts to 44kDa in lane 2, which contains the protein extracted from leaves infiltrated with *TaU4* cloned into *pEarleyGate104*. TaU4 is 17kDa, the same size as the difference between the band sizes in lane 1 and 2, therefore proving that the cellular localization seen in Figure 17b is due to YFP:TaU4 protein not YFP only.

Figure 17:

(a) **(b)**



(c)

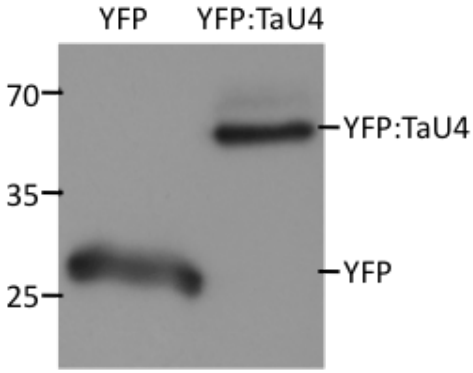


Figure 17: TaU4 cellular localization. (a) Confocal microscopy of the cellular localization of YFP only control (pEarleyGate 104) in *N. benthamiana* 4 days after infiltration. **(b)** Confocal microscopy of the cellular localization of YFP:TaU4 (pEarleyGate 104) in *N. benthamiana* 4 days after infiltration. **(c)** Total protein was extracted from infiltrated *N. benthamiana* of both YFP and YFP:TaU4 and separated on an SDS PAGE gel. A western blot of the protein bands was then visualized using anti-YFP. YFP = 27kDa and YFP:TaU4 = 44kDa.

5. A study into the roles of four E2 enzymes in the wheast-Septoria interaction

5.1. Introduction

The previous study (Lee et al unpublished) that identified TaU4 as having a potential involvement in Septoria-wheat interaction also identified four other wheat E2 enzymes whose expression changed in leaves after Septoria infection. These four TaU enzymes were designated TaU1, TaU2, TaU3 and TaU5. The coding sequence and fragments for silencing are shown in the appendix. For the genes *TaU1*, *TaU2* and *TaU5* the two independent specific fragments for silencing had already been cloned into *BSMV* and transformed into agrobacterium. The same techniques as used for *TaU4* were employed for investigating *TaU1*, *TaU2*, *TaU3* and *TaU5*.

This chapter aims to find other promising E2 enzyme for studying in more depth with regards to the Septoria-wheat interaction. Different E2 enzymes may function to either initiate or elongate the formation of ubiquitin chains or they can function in both initiation and elongation (Verma et al 1997; Li et al 2007; Windheim et al 2007). Investigating multiple E2 enzymes may suggest pairs of wheat E2 enzymes that work together in plant defence against Septoria. If potential partners for TaU4 are found then silencing of both E2 enzymes could be performed in the future leading to more understanding of how the family E2 enzymes function in defence.

5.2. *TaU1*, *TaU2*, *TaU3* and *TaU5* alignment against known E2 enzymes

The wheat E2 enzyme protein sequences were compared to known human E2 enzyme protein sequences that have previously been assigned to a class (I-IV) based on their length and structure around the UBC fold (Figure 2). An alignment

of the E2 enzyme protein sequences was constructed using Clustlaw omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Figure 18a). The active site cysteine is highlighted in black, there is a higher degree of homology surrounding the active site cysteine than elsewhere in the protein amino acid sequence. E2 enzymes in class IV have noticeably more amino acids, with regions that are not present in any of the other classes (at the N and C terminals, which are not shown in Figure 18a). A phylogenetic tree was constructed using Clustlaw omega (Figure 18b), which shows that TaU1-3 are all very homologous and most closely related to class I E2 enzymes that just have the UBC fold and no terminal extensions. TaU1, TaU2 and TaU3 enzymes are all small proteins, with 148, 148 and 118 amino acids and a molecular weight of 16.5kDa, 16.5 kDa and 13.5kDa respectively. TaU1-3 also share homology with TaU4. TaU5 is the least homologus to the other four wheat E2s, showing more homology to class II and III E2 enzymes rather than class I. It is a slightly larger enzyme with 183 amino acids and a molecular weight of 20.9kDa.

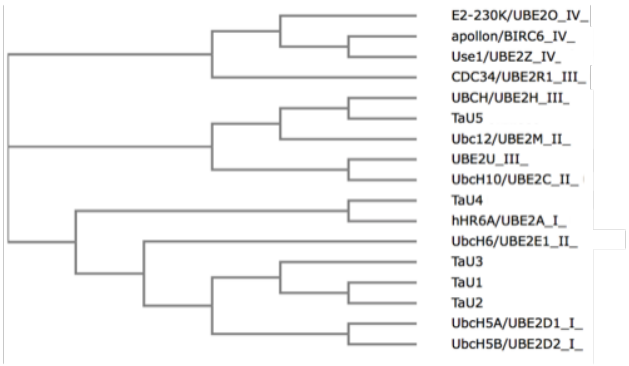
The protein sequences of the wheat E2 enzymes were also aligned with others whose function has previously been studied, with some having a role in defence (OsUBC5b and FcUBC). There is a higher degree of homology within the protein sequence, especially around the active site, with these E2 enzymes than the classified human E2 enzymes (Figure 18c). A phylogenetic tree was also generated to compare the homology of these enzymes (Figure 18d). This phylogenetic tree shows that TaU5 and TaU4 are more homologous than the previous phylogenetic tree suggested. They are both closely related to *A. thaliana* E2 enzymes involved in the timing of flowering (AtUBC1 and AtUBC2) and cell cycle regulation (AtUBC19). From this tree it appears that TaU4 and TaU5 are also closely related to TaU3, and that TaU3 is a possible evolutionary predecessor of TaU4 and TaU5. TaU1 and TaU2 are again closely related and appear to be predecessors of the other E2 enzymes (Figure 18d).

Figure 18:

(a)

```
E2-230K/UBE2O(IV)   1013 PAVPPHFCYLS--QCSGRLNPNLYD-NGKVCVSLLGTWIGK------GTERWTSKSSL-L 1062
apollon/BIRC6(IV)   4637 PSSPPLVNLETTGGHSVRFNPNLYN-DGKVCLSILNTWHG------RPEEKWNPQTSSFL 4689
UBCH/UBE2H(III)       62   PFKSPSIGFMN-----KIFHPNIDEASGTVCLDVIN-------------QTWTALYDL-T 102
TaU5                  60   PYKSPSIGFIN-----KIYHPNVDEMSGSVCLDVIN-------------QTWSPMFDL-V 100
UBE2U(III)            64   NYAPPVVKFIT-----IPFHPNVDPHTGQPCIDFLDNP-----------EKWNTNYTL-S 106
Ubc12/UBE2M(II)       87   PHDPPKVKCET-----MVYHPNIDL-EGNVCLNILR-------------EDWKPVLTI-N 126
CDC34/UBE2R1(III)     69   PYSPPAFRFLT-----KMWHPNIYE-TGDVCISILHPPVDDPQSGELPSERWNPTQNV-R 121
Use1/UBE2Z(IV)       159   PIHPPRVKLMTTGNNTVRFNPNFYR-NGKVCLSILGT---------WTGPAWSPAQSI-S 207
UbcH10/UBE2C(II)      90   PYNAPTVKFLT-----PCYHPNVDT-QGNICLDILK-------------EKWSALYDV-R 129
TaU4                  64   PNKPPTVRFIS-----RMFHPNIYA-DGSICLDILQ-------------NQWSPIYDV-A 103
hHR6A/UBE2A(I)        64   PNKPPTVRFVS-----KMFHPNVYA-DGSICLDILQ-------------NRWSPTYDV-S 103
UbcH6/UBE2E1(II)     107   PFKPPKVTFRT-----RIYHCNINS-QGVICLDILK-------------DNWSPALTI-S 146
TaU3                  32   PFKPPKVAFRT-----KVFHPNINS-NGSICLDILK-------------DQWSPALTI-S 71
TaU1                  61   PFKPPKVSFKT-----KVFHPNINS-NGSICLDILK-------------EQWSPALTI-S 100
TaU2                  61   PFKPPKVSFKT-----KVFHPNINS-NGSICLDILK-------------EQWSPALTI-S 100
UbcH5A/UBE2D1(I)      61   PFKPPKIAFTT-----KIYHPNINS-NGSICLDILR-------------SQWSPALTV-S 100
UbcH5B/UBE2D2(I)      61   PFKPPKVAFTT-----RIYHPNINS-NGSICLDILR-------------SQWSPALTI-S 100
```

(b)



(c)

```
AtUBC1     63 YPNKPPTVRFVSRMFHPNIYA-DGSICLDILQNQWSPIYDVAAILTSIQ-SLLCDPNPNSPANS 124
AtUBC2     63 YPNKPPTVRFVSRMFHPNIYA-DGSICLDILQNQWSPIYDVAAILTSIQ-SLLCDPNPNSPANS 124
TaU4       63 YPNKPPTVRFISRMFHPNIYA-DGSICLDILQNQWSPIYDVAAILTSIQ-SLLCDPNPNSPANS 124
AtUBC19    95 YPFKSPKVKFETCCFHPNVDL-YGNICLDILQDKWSSAYDVRTILLSIQ-SLLGEPNISSPLNN 156
TaU1       60 YPFKPPKVSFKTKVFHPNINS-NGSICLDILKEQWSPALTISKVLLSIC-SLLTDPNPDDPLVP 121
TaU2       60 YPFKPPKVSFKTKVFHPNINS-NGSICLDILKEQWSPALTISKVLLSIC-SLLTDPNPDDPLVP 121
OsUBC5b    60 YPFKPPKVALKTKVFHPNINS-NGSICLDILKEQWSPALTISKVLLSIC-SLLTDPNPDDPLVP 121
OsUBC      60 YRFNRPKVAFKTKVFHPNINS-NGSICLDILKEQWSPALTVSKVLLSIC-SLLTVPNPDDPLVP 121
TaU3       31 YPFKPPKVAFRTKVFHPNINS-NGSICLDILKDQWSPALTISKVLLSIC-SLLCDPNPDDPLVP 92
FcUBC      61 YPFQPPQIKFKTPIYHMNVGP-YGDICLDILDKNWSPALSISKVLLVIC-VLMTDPNPDDPLRF 122
AtCOP10    95 YPFKPPKLVFKTRIYHCNVDT-AGDLSVNILRDSWSPALTITKVLQAIR-SIFLKPEPYSPALP 156
LeUBC1     62 YPFEPPKMKFATKVWHPNISSQS-AICLDILKDQWSPALTLKTALLSIQ-ALLSAPEPDDPQDA 124
TaU5       62 YPYKSPSIGFINKIYHPNVDEMSGSVCLDVINQTWSPMFDLVNVFEVFLPQLLLYPNPSDPLNG 123
```

(d)

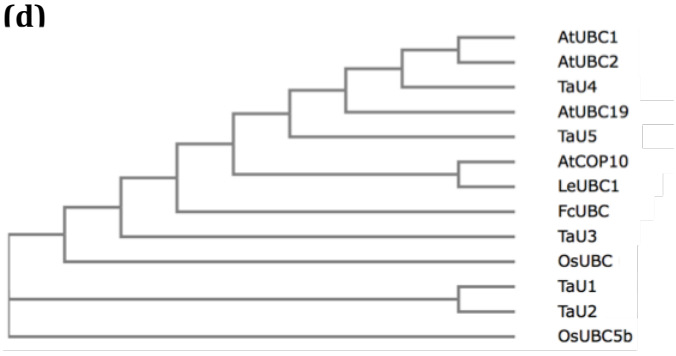


Figure 18: TaU1, TaU2, TaU3 and TaU5 protein sequences were aligned to other known E2 enzymes protein sequences using Clustlaw omega. (a) TaU1, TaU2, TaU3 and TaU5 protein sequences were aligned to human E2 enzymes protein sequences that have already been assigned a class (Class I-IV) based on their structure. **(b)** A phylogenetic tree was generated using Clustlaw omega comparing protein sequences of the human E2's to TaU1, TaU2, TaU3 and TaU5 to show closely related E2 enzymes. **(c)** Protein sequences of TaU1, TaU2, TaU3 and TaU5 were aligned to E2 enzymes whose function has been defined, including E2 enzymes involved in defence. **(d)** A phylogenetic tree was generated to compare the protein sequences of known E2 enzymes to TaU1, TaU2, TaU3 and TaU5. At = *Arabidopsis thaliana*, Ta = *Triticum aestivum*, Os = *Oryza sativa*, Le = *Lycopersicon esculentum* and Fc = *Fenneropenaeus chinensis*. Accession numbers for the sequences used in this figure are: E2-230K/UBE2O = NM_022066.3, apollon/BIRC6 = NM_016252.3, UBCH/UBE2H = BC006277.2, UBE2U = BC029895.1, Ubc12/UBE2M = NM_003969.3, CDC34/UBE2R1 = NM_152489.1, Use1/UBE2Z = NM_004359.1, UbcH10/UBE2C = BC007656.2, hHR6A/UBE2A = DQ068065.1, UbcH6/UBE2E1 = BC009139.1, UbcH5A/UBE2D1 = BC015997.1, UbcH5B/UBE2D2 = BC033349.1, AtUBC1 = , AtUBC2 = , AtUBC19 = AY127573.1, AtCOP10 = , LeUBC1= X82938.1, FcUBC = GU723296, OsUBC = U15971.1 and OsUBC5b = AB074412.1.

5.3. Measuring expression levels of *TaU1*, *TaU2*, *TaU3* and *TaU5* in Septoria infected and healthy wheat tissue

The expression levels of each of the other *TaU* genes were tested by real time PCR from Septoria infected tissue and healthy tissue at 14 days post infection. This time point was chosen as it represents the onset of visible phenotype of the Septoria infection and the switch to necrotrophic growth (Figure 19). The real time PCR primers were tested with serial dilutions of cDNA (appendix) and the PCR products were separated using gel electrophoresis to check there was only one band therefore only one gene being amplified. Again two independent specific primer sets for each gene were used to avoid amplifying the gene fragment from the *BSMV* vector instead of the mRNA produced by the plant. However after checking with the serial dilutions 1B primers were discarded and the primers for 2B were discarded as they produced multiple amplified DNA fragments so would produce unreliable results on the real time PCR. The leaf samples and the qPCRs were repeated once in this study due to time constraints.

The expression levels for *TaU1* and *TaU3* both increase by over 100% in the infected wheat leaves compared to the healthy wheat leaves (Figures 19a and 19c). Similar to *TaU4*, *TaU2* shows a reduction in expression in infected wheat leaves of over 50% (Figure 19b). *TaU5* expression was measured with two different primer sets; 5A primers found small increase in expression (Figure 19d) whereas 5B primers found a decrease in expression of over 300% (Figure 19e).

Figure 19:

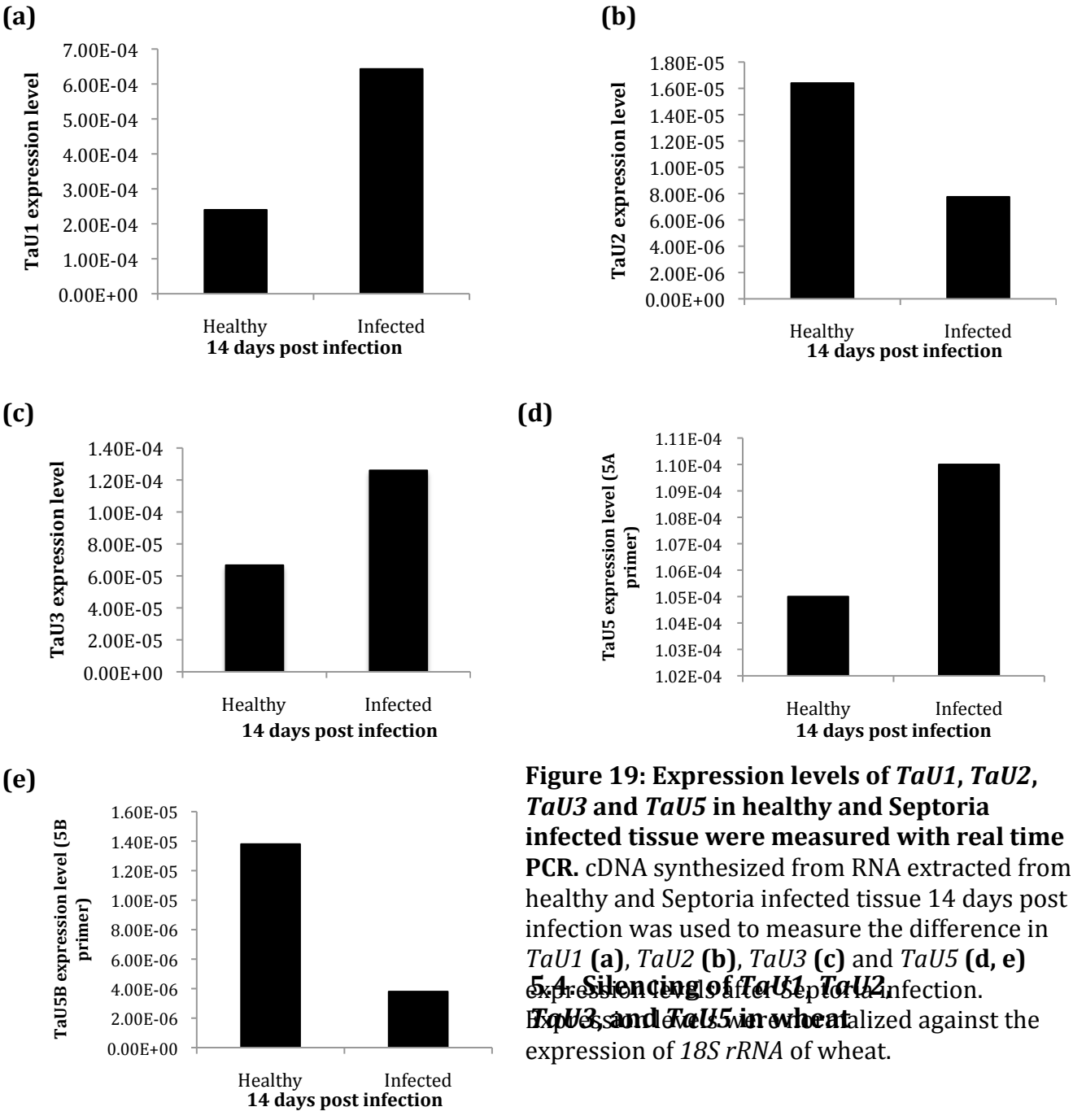


Figure 19: Expression levels of *TaU1*, *TaU2*, *TaU3* and *TaU5* in healthy and Septoria infected tissue were measured with real time PCR. cDNA synthesized from RNA extracted from healthy and Septoria infected tissue 14 days post infection was used to measure the difference in *TaU1* **(a)**, *TaU2* **(b)**, *TaU3* **(c)** and *TaU5* **(d, e)** expression levels after Septoria infection. Expression levels were normalized against the expression of *18S rRNA* of wheat.

5.4. Silencing of *TaU1*, *TaU2*, *TaU3*, and *TaU5* in wheat

To test the silencing efficiency of the *TaU* genes RNA was extracted from *BSMV:TaU1A*, *BSMV:TaU1B*, *BSMV:TaU2A*, *BSMV:TaU2B*, *BSMV:TaU3B*, *BSMV:TaU5A*, *BSMV:5B,* Mock and *BSMV:00* silenced wheat (at the onset of silencing) for cDNA synthesis. The mRNA levels of each gene were then measured using real time PCR, which was only repeated once due to time constraints.

To test for silencing within *BSMV:TaU1A* and *BSMV:TaU1B* silenced wheat the primer 1A was used, which amplifies within *TaU1A* silencing fragment. *BSMV:00* and Mock silenced wheat leaves both have similar levels of *TAU1*, as shown in Figure 20a. Figure 20a shows that *TaU1* was silenced in *BSMV:TaU1B* when compared to Mock and *BSMV:00* silenced controls.

There is an increase in *TaU1* expression in *TaU1A* silenced wheat of 350% and 363% compared to *BSMV:00* and Mock silenced wheat leaves respectively (Figure 20a a). The 1A primer used in the real time PCR amplifies within the *TaU1A* silencing fragments, which may explain the higher than expected levels of *TaU1A* expression in *BSMV:TaU1A* silenced leaves as it could be amplifying viral encoded *TaU1A* fragment RNA.

There is a difference in the expression levels of *TaU2* found between Mock and *BSMV:00*, with Mock having 80% more *TaU2* than *BSMV:00* (Figure 20b). *BSMV:TaU2A* silenced wheat leaves have been silenced by 84.8% and 91.7% compared to *BSMV:00* and Mock silenced wheat respectively. *BSMV:TaU2B* shows less silencing than *BSMV:TaU2A* silenced wheat leaves, with an increase in expression of 8% compared to *BSMV:00* and a decrease in expression of 40.8% when compared to Mock silenced wheat (Figure 20b).

TaU3B expression has been silenced within the *BSMV:TaU3B* silenced wheat compared to *BSMV:00* and Mock silenced wheat leaves as shown in Figure 20c. The expression levels of *TaU3* between *BSMV:00* and Mock differ by 24%, with *BSMV:00* having a slightly higher *TaU3* expression level. *TaU3B* has been silenced by 75.4% and 80.3% in *BSMV:TaU3B* silenced wheat leaves compared to that of *BSMV:00* and Mock silenced wheat (Figure 20c).

The expression levels of *TaU5* were measured by real time PCR using 5A and 5B primers (amplifying a region within the silencing fragments *TaU5A* and *TaU5B* respectively) in both *BSMV:TaU5A* and *BSMV:TaU5B* silenced wheat leaves. Figure 20d shows there to be a 261% increase of *TaU5* expression, when measured with 5A primers, between Mock to *BSMV:00* controls, however this only represents a small fold increase of 6.60E-04. *BSMV:TaU5A* silenced wheat leaves have higher mRNA levels than *BSMV:TaU5B* when measuring with 5A primers. Compared to *BSMV:00*, *TaU5* has been silenced by 62.9% and 91.8% in *BSMV:TaU5A* and *BSMV:TaU5B* silenced wheat leaves respectively using 5A primers (Figure 20d). When comparing *TaU5* expression levels to Mock, *BSMV:TaU5A* has an increase of 34% increase and *BSMV:TaU5B* has a decrease of 70.2% (Figure 20d) when using 5A primers. This increase in *TaU5* expression in *BSMV:TaU5A* silenced wheat leaves compared to the Mock control could again be the viral encoded *TaU5A* fragment being amplified by the 5A primers during the PCR experiment.

Using 5B primers there is a 62% increase in *TaU5* expression between Mock to *BSMV:00* silenced wheat, representing an increase of only 3.59E-05 (Figure 20e). *BSMV:TaU5B* silenced wheat leaves show an increase in expression of *TaU5*, when measured with 5B primers, of 243.6% and 30.2% compared to *BSMV:00* and Mock respectively (Figure 20e), again this could be due to the amplification of viral encoded *TaU5B* fragment. *BSMV:TaU5A* silenced wheat leaves measured for silencing with 5B primers shows a low level of silencing when compared to *BSMV:00* (19.8%) but an increase in expression when compared to Mock (30.2%) (Figure 20e).

Figure 20:

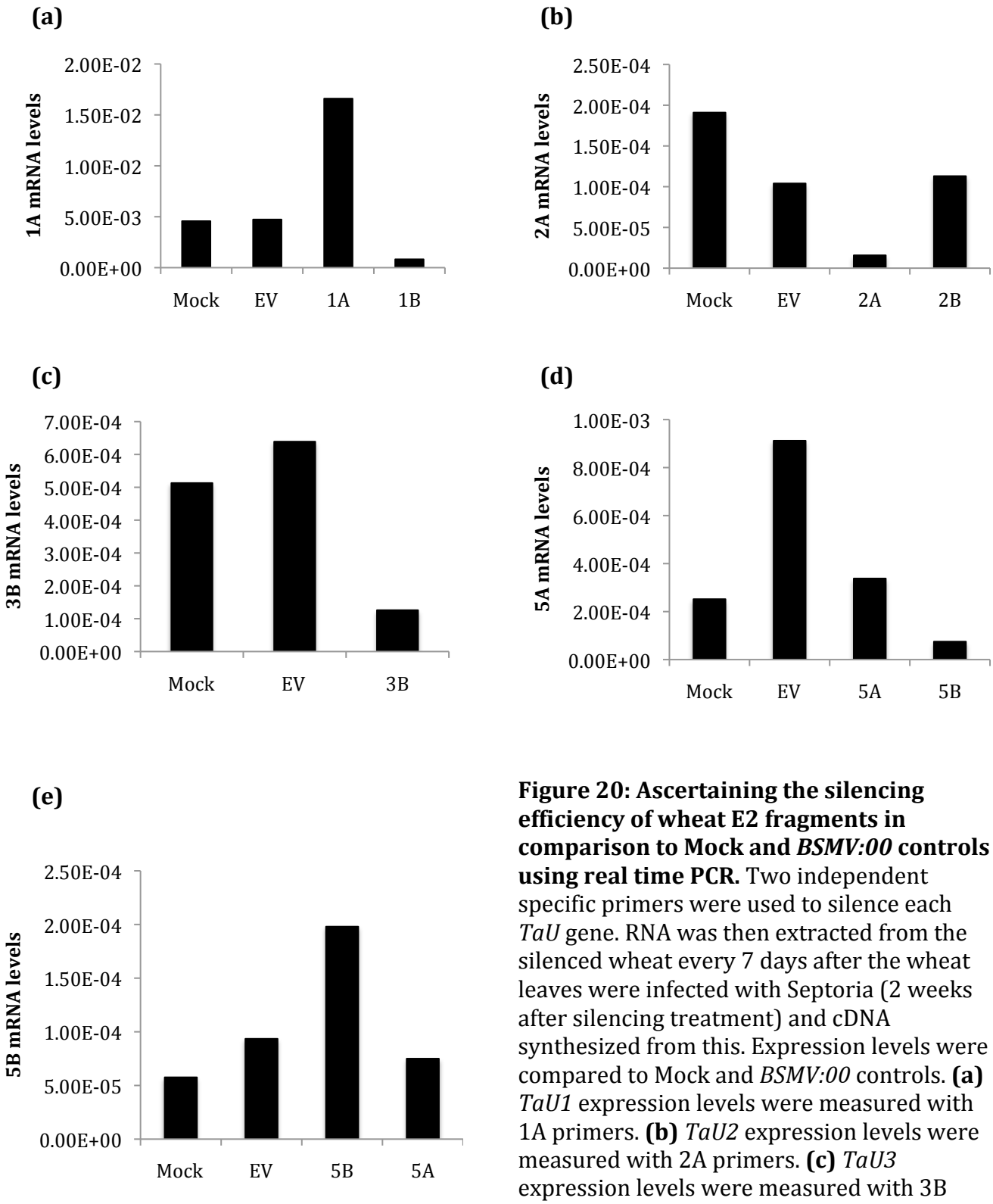


Figure 20: Ascertaining the silencing efficiency of wheat E2 fragments in comparison to Mock and *BSMV:00* controls using real time PCR. Two independent specific primers were used to silence each *TaU* gene. RNA was then extracted from the silenced wheat every 7 days after the wheat leaves were infected with Septoria (2 weeks after silencing treatment) and cDNA synthesized from this. Expression levels were compared to Mock and *BSMV:00* controls. **(a)** *TaU1* expression levels were measured with 1A primers. **(b)** *TaU2* expression levels were measured with 2A primers. **(c)** *TaU3* expression levels were measured with 3B primers. **(d)** *TaU5* expression levels were measured with 5A primers and **(e)** a second *TaU5* primer, 5B. Expression levels were normalized against the expression of *18S rRNA* of wheat.

5.5. Septoria infection upon *BSMV:TaU1*, *BSMV:TaU2*, *BSMV:TaU3* and *BSMV:TaU5* silenced wheat

The symptoms of the silenced Septoria infected wheat leaves were followed over 28 days. Septoria infection depends on the environment especially humidity (Eyal et al 1987), if there was any difference in the environment which Septoria had been grown it could effect the infectivity of the Septoria. This could possibly explain why there was a higher severity of Septoria infection phenotype on this experiment as opposed to previous infection experiments (Figure 7). The *BSMV:00* silenced wheat shows less severe disease symptoms at 14dpi compared to the Mock silenced wheat, which has necrosis over the entire leaf (Figure 21). By 21dpi the Mock leaves have darkened in colour even more and there is more necrosis appearing, very similar to the leaves infected after 28 days with Septoria (Figure 21f). *BSMV:00* silenced wheat also has full leaf necrosis, but has slightly less severe disease symptoms than the Mock control (Figure 21e).

Both *BSMV:TaU1A* and *BSMV:TaU1B* silenced wheat have severe disease symptoms by 14dpi, with necrosis over the entire leaf. By 21dpi the leaves have become fully necrotic similar to that of 28dpi. *BSMV:TaU1A* and *BSMV:TaU1B* show more disease symptoms than either Mock or *BSMV:00* silenced wheat (Figure 21a).

Similarly to *BSMV:TaU1A* and *BSMV:TaU1B* the wheat silenced with *BSMV:TaU2A* and *BSMV:TaU2B* has more symptoms than either *BSMV:00* or Mock silenced wheat. At 14dpi there is necrosis over most of the leaf, with *BSMV:TaU2A* starting to show symptoms at the edges of the leaf at 7dpi. *BSMV:TaU2B* has less severe symptoms than *BSMV:TaU2A*; at 14dpi the *BSMV:TaU2B* wheat retains a healthier green colouring than *BSMV:TaU2A* that has an orange/brown colouring. Full necrosis is visible for both *BSMV:TaU2A* and *BSMV:TaU2B* at 21dpi (Figure 21b).

BSMV:TaU3B silenced wheat appears to be the most resistant to Septoria infection. At 14dpi the leaf still has almost 50% green colouring, compared to the

Mock control, which had no green colouring at 14dpi and *BSMV:00* that had approximately 25% of green leaf tissue still (Figure 21c).

BSMV:TaU5A and *BSMV:TaU5B* both appear more resistant to Septoria infection, however less so than *BSMV:TaU3B*. Although both have very little to no green colouring at 14dpi, they are less necrotic when compared to Mock and *BSMV:00* control silenced wheat (Figure 21d).

Figure 21:

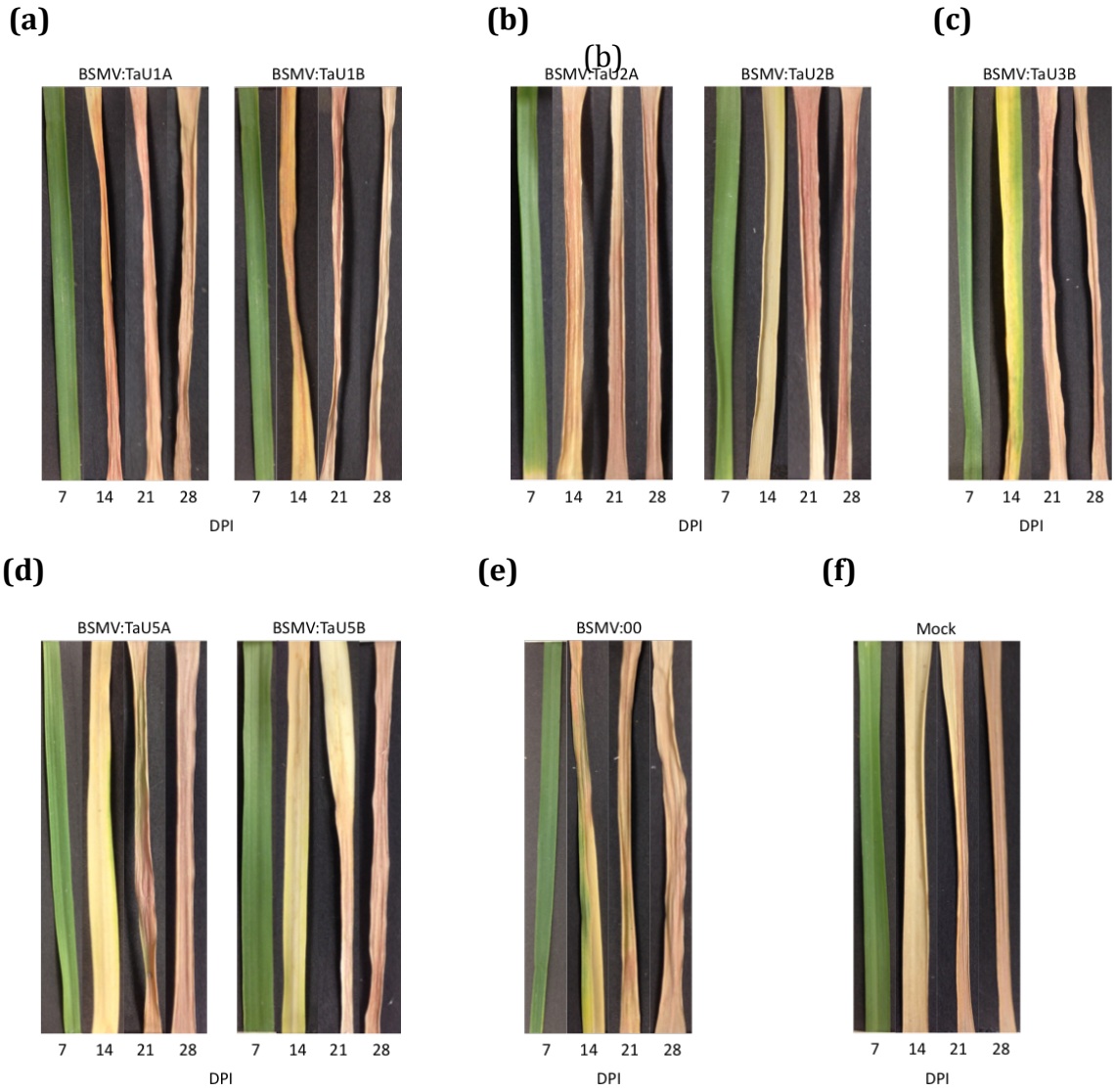


Figure 21: Four *TaU* silenced wheat leaves after infection with Septoria fungus. Pictures are of wheat silenced with *BSMV:TaU1A*, *BSMV:TaU1B* **(a)**, *BSMV:TaU2A*, *BSMV:TaU2B* **(b)**, *BSMV:TaU3B* **(c)**, *BSMV:TaU5A*, *BSMV:TaU5B* **(d)**, *BSMV:00* **(e)** and Mock **(f)**. White light pictures were taken every 7 days up until the end of the experiment (28 days) to follow the disease symptoms of the Septoria and variations between the symptoms for the different silenced wheat leaves. DPI = Days post infection.

5.6. Pcynidia counts for *BSMV:TaU1*, *BSMV:TaU2*, *BSMV:TaU3* and *BSMV:TaU5* silenced wheat after 28 days of Septoria infection

The pcynidia were counted over a 2cm length of the infected leaves. These leaves were then used for the spore count. Putting the wheat leaves into high humidity conditions induced pcynidia and spore production.

The pycnidia count for the *BSMV:00* control was lower than that from the experiments with *TaU4* silenced wheat (Figure 8). The pycnidia count between *BSMV:00* and Mock silenced control is not significantly different (student t test = 0.33), with a large overlap in the standard deviation (indicated by the error bars) as be seen in Figure 22.

Wheat silenced with *BSMV:TaU1A* and *BSMV:TaU1B* fragments both have an average pcynidia count in-between the two controls (*BSMV:00* and Mock), they both have smaller standard deviations than either of the controls, showing that there is less variation between the pcynidia counts (Figure 22a). However neither is significantly different from either *BSMV:00* or Mock infected wheat.

BSMV:TaU2A and *BSMV:TaU2B* silenced wheat also have an average pcynidia count that is lower than *BSMV:00* but higher than Mock silenced wheat (Figure 22b). Again neither is significantly different to either of the controls in a student t test.

The average pcynidia count for *BSMV:TaU3B* silenced wheat is above that of both *BSMV:00* and Mock silenced wheat, 31.8, 30.9 and 20.1 pcynidia respectively. There is however a large cross over with the error bars (1 standard deviation of the mean) and *BSMV:TaU3B* pycnidia count is not significantly different to that of *BSMV:00* or Mock silenced wheat (Figure 22c).

Wheat silenced with *BSMV:TaU5A* and *BSMV:TaU5B* both show a higher average pcynidia count than either of the controls. *BSMV:TaU5B* silenced wheat has a higher average count than *BSMV:TaU5A*. The counts for *BSMV:TaU5A* are not significantly different to the two controls, whereas *BSMV:TaU5B* pycnidia count

is significantly different to Mock silenced wheat count (student t test value $p<0.05$) but not to *BSMV:00* count (0.131) (Figure 22d).

Figure 22:

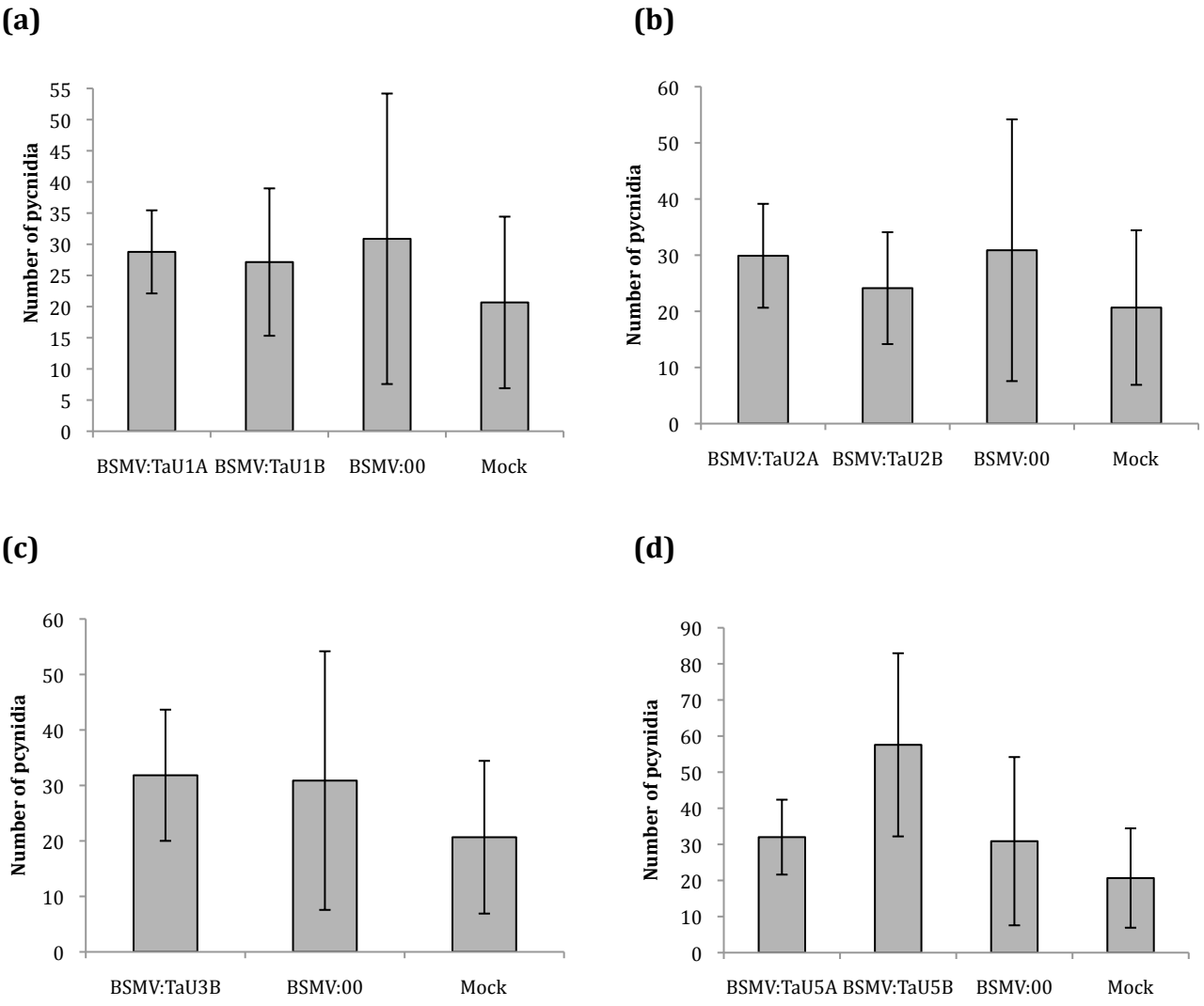


Figure 22: Pcynidia count for 28 day Septoria infected *TaU1*, *TaU2*, *TaU3* and *TaU5* silenced wheat leaves. (a) *BSMV:TaU1A*, *BSMV:TaU1B*, **(b)** *BSMV:TaU2A*, *BSMV:TaU2B*, **(c)** *BSMV:TaU3B*, **(d)** *BSMV:TaU5A* and *BSMV:TaU5B* silenced wheat were all compared to *BSMV:00* and Mock silenced wheat. The pcynidia were counted over 2cm length of the leaf. 1 technical repeat and 5 biological repeats were performed. The error bars represent 1 standard deviation.

5.7. Spore counts for *BSMV:TaU1*, *BSMV:TaU2*, *BSMV:TaU3* and *BSMV:TaU5* silenced wheat after 28 days of Septoria infection

The spore counts were much lower than those from the experiments with *TaU4* silenced wheat (Figure 8), the average count for the *BSMV:00* silenced wheat is 2.94E+06 spores per ml compared to 3.32E+07 spores per ml in the experiments with *TaU4*. There is also a major difference between the two controls, *BSMV:00* and Mock, with a student t test value of $p<0.05$, showing a high statistical significance. With this in mind these results may not be accurate, as the spores have not developed to the levels that have previously been found possible (Figure 23).

BSMV:TaU1A, which caused the production of less pycnidia than *BSMV:00* silenced wheat, has produced more spores on average compared to *BSMV:00* and Mock (student t test value $p<0.05$). *BSMV:TaU1B* has a slightly lower average spore count than *BSMV:00* (by 3.8E+05 spores per ml) but there was a wide range (1.00E+06-6.00E+06 spores per ml) in the number of spores per ml that it is not statistically different to either the *BSMV:00* or the Mock silenced wheat (Figure 23a).

Similar to the results from the pycnidia count, *BSMV:TaU2A* has an average spore count lower than *BSMV:00* but higher than Mock silenced wheat. There was a high level of consistency between biological repeats from the spore counts of *BSMV:TaU2A* silenced wheat (standard deviation = 1.5) and it is significantly higher than Mock ($p<0.05$) according to student t tests but on average lower than *BSMV:00* . *BSMV:TaU2B* has a higher spore count than either of the controls, and unsurprisingly is significantly different to the spore count from Mock silenced wheat (student t test value $p<0.05$) but not to the *BSMV:00* count (Figure 23b).

As with the pycnidia count, the spore count average for *BSMV:TaU3B* silenced wheat is higher than that from either *BSMV:00* or Mock controls, with on average almost 50% more spores per ml than *BSMV:00* (5.56E+06 and 2.94E+06 for *BSMV:TaU3B* and *BSMV:00* respectively). However due to a relatively wide range

in the spore counts there is not a significant difference under a student t test between *BSMV:00* and *BSMV:TaU3B* silenced wheat spore counts (p=0.140) (Figure 23c).

Following the pattern of the pycnidia count, *BSMV:TaU5A* has a lower average number of spores than *BSMV:00* but higher average than Mock. *BSMV:TaU5B* has a higher average number of spores per ml than both of the controls. *BSMV:TaU5B* has over double the average number of spores per ml than *BSMV:00* (6.69E+06 and 2.94E+06 per ml respectively) and is statistically significant ($p<0.05$) according to a student t test compared to both controls and *BSMV:TaU5A* spore counts. *BSMV:TaU5A* spore counts are not statistically significant under a student t test compared to *BSMV:00* but is compared to Mock silenced wheat ($p<0.05$) (Figure 23d).

Figure 23:

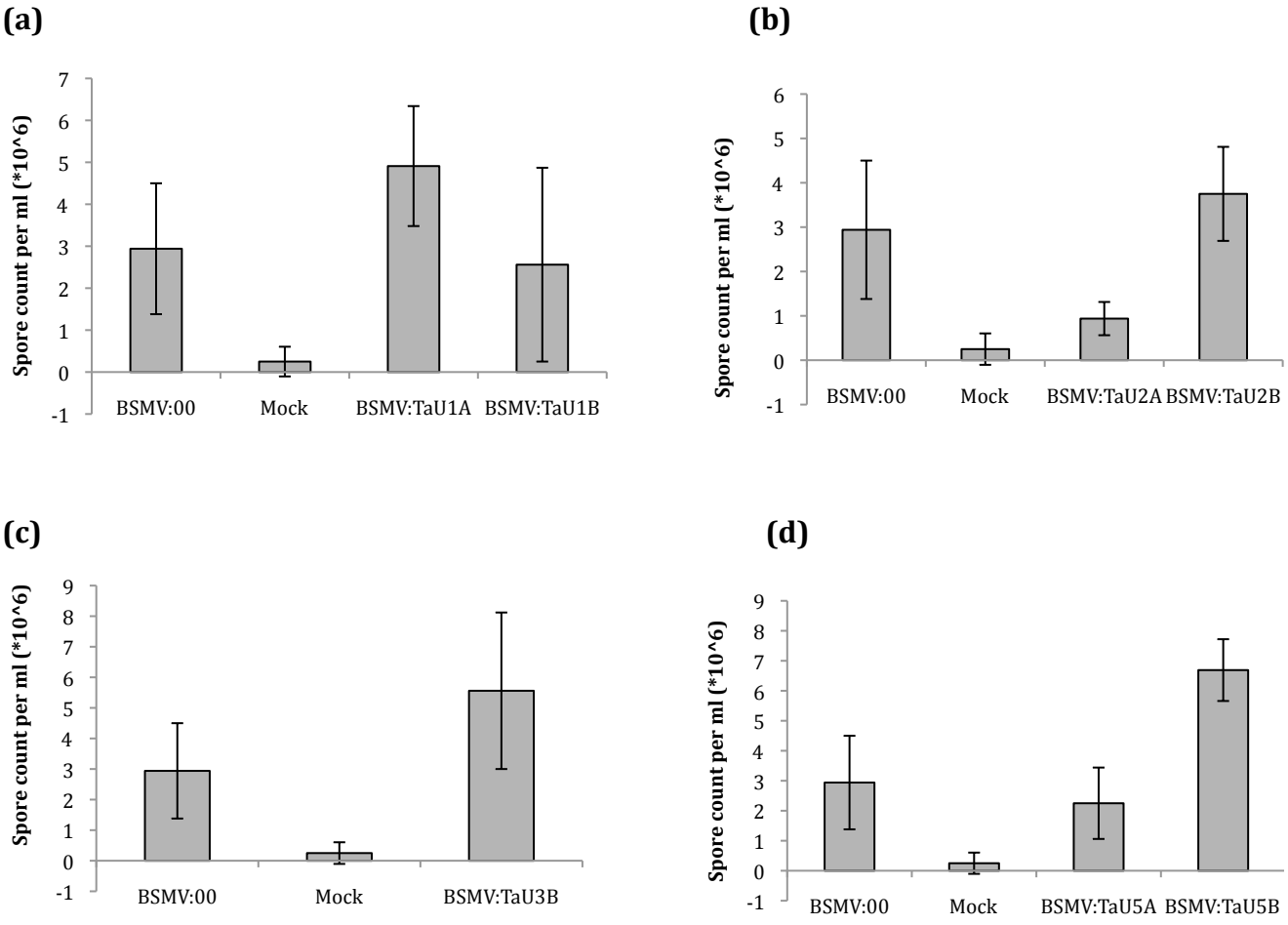


Figure 23: Septoria spore count from wheat silenced with E2 enzymes. The spore counts from wheat silenced with *BSMV:TaU1A*, *BSMV:TaU1B* **(a)**, *BSMV:TaU2A*, *BSMV:TaU2B* **(b)**, *BSMV:TaU3B* **(c)**, *BSMV:TaU5A* and *BSMV:TaU5B* **(d)** were all compared to the spore counts from Mock and *BSMV:00* controls. The spores were measured using a haemocytometer slide and averaged across the 4 boxes of the slide. This was then used to work out the spores per ml (*10^6). This graph represents 1 technical repeats and 10 biological repeats. Error bars represent 1 standard deviation of the mean.

6. Discussion

6.1. *TaU4* gene expression changes after Septoria infection

Measurements of the gene expression of *TaU4* show there to be more *TaU4* expression in the infected tissue than in healthy wheat tissue (Figure 4). However just before the disease symptoms occur and the levels of *60S L13* Septoria expression increase rapidly (16dpi, Figure 9), the expression levels of *TaU4* decrease in the infected tissue compared to the healthy tissue. This also happens just before the second large increase in Septoria *60S L13* expression in infected wheat at 28dpi. In Figure 4 at 24 and 26dpi there is a rise in *TaU4* mRNA levels in the healthy wheat but the infected wheat keeps the expression lower.

One possible explanation for this is that *TaU4* is a negative regulator of defence, therefore when the plant detects Septoria it suppresses *TaU4* expression. Vargas et al (2012) suggested one cue for the switch from biotrophic to necrotrophic growth in the hemibiotrophic fungi *Colletotricum graminicola,* which infects maize, could be that the fungus is trying to avoid the accumulated plant defence. When the fungus detects plant defence response it switches from biotrophic to necrotrophic growth, overcoming the plants defences.

If the target of TaU4 ubiquitination was a positive regulator of defence that accumulated over time but was broken down periodically by TaU4 targeting it to the 26S proteosome, it may explain why *TaU4* expression increases every 4-6 days (Figure 4). The plant is priming itself for a quick defence response by building up a low level of the positive defence regulator, but when no pathogens are detected *TaU4* expression is switched on to breakdown the positive regulator. Then, when the plant senses the Septoria fungus it represses the expression of *TaU4*, leading to an increase in the accumulation of the potential positive regulator of defence. The Septoria may then sense the wheat's defence building, causing it quickly switch to necrotrophic growth. This could also explain the difference in *TaU4* expression between healthy and infected wheat just before the end of Septoria's life cycle in the wheat leaf, as again the *TaU4* is

suppressed in infected wheat compared to healthy wheat that then correlates with an increase in Septoria *60S L13* expression 2 days later, at 28dpi (Figures 4 and 9). However this explanation does not explain why in general the *TaU4* expression is higher in infected wheat than healthy wheat. The cue for the switch between biotrophic to necrotrophic growth was also suggested after studying *C. graminicola* hemibiotroph that infects intracellulary (Vargas et al 2012), unlike Septoria infection that stays in the apoplastic extracellular space (Eyal et al 1987). This would also explain the Septoria infection phenotype seen in *BSMV:TaU4A* and *BSMV:TaU4B* silenced wheat as the visible disease symptoms are delayed in both lines of *TaU4* silenced wheat in comparison to *BSMV:00*. If *TaU4* is constantly suppressed (by the silencing), it may allow the potential positive regulator of defence to accumulate and increase the wheat defence against Septoria. This would cause the Septoria to grow slower in the wheat due to the heighted defence response, taking it longer to develop to the point where it could read into the wheat defence profile and use this as a cue to switch between biotrophic and necrotrophic growth.

One difference that could affect the healthy leaf samples is that it was not ensured that the same leaf (i.e. 4th leaf) was collected each time (the 3rd-5th of the infected tissue samples were collected each time), instead a couple of leaves from different developmental stages were collected ranging from the 3rd-8th leaf.

This is potentially important, as ubiquitination has been implicated in plant development. If the collected leaves were at different developmental stages it may impact on the ubiquitin machinery profile hence changing *TaU4* expression. For instance, after auxin application the E3 ligase SINAT5 is upregulated in *A. thaliana*. SINAT5 controls lateral root development by targeting the positive regulator of lateral root development, NAC1, to the 26S proteosome for degradation (Xie et al 2002). Auxin gradients control many plant development processes including shade avoidance in leaves (Tao et al 2005), gravitropism responses in both leaves and roots (Vanneste and Friml 2009) and cell elongation (Evans 1984). One way these developmental processes controlled by auxin is through the binding of auxin to the E3 ligase TIR1 (transport inhibitor response 1) F-box protein (part of an E3 ligase SCF, SKP1-CUL1-F-box protein

complex) that promotes the 26S proteosomal degradation of the transcriptional repressor/activator AUX/IAA protein family (Dharmasiri et al 2005). Ubiquitination also controls plant development independently of auxin, for instance organ growth is repressed by a ring E3 ligase called BIG BROTHER (BB) thought to ubiquitinate and therefore target growth stimulators for degradation by the 26S proteosome (Disch et al 2006).

6.2. Silencing of *TaU4* in wheat

As seen in Figure 6 silencing of *TaU4* appears to be incomplete when measured with real time PCR, however due to the phenotypic changes (Septoria infection, pycnidia and spore counts) observed in BSMV:TaU4A and BSMV:TaU4B silenced wheat it indicates that silencing has occurred in the Septoria infected leaves. There are many possible factors that could have led to apparent lack of silencing these are described below and would be taken into account for future work.

Firstly more silencing experiments need to be performed and repeats of the real time experiments done so that the outcome can be more reliable. As well as this the silencing needs to be measured at more frequent intervals. This is based on the expression patterns of TaU4 (figure 4) that show oscillations in the expression every 4-6 days. The silencing samples for *BSMV:TaU4A* and *BSMV:TaU4B* could have been collected at a time where TaU4 expression was in a peak and that the *BSMV:00* silenced wheat was going through a dip in the expression at the times of sample collection. If more frequent samples were collected then the oscillations in the TaU4 expression could be followed at the same time as the silencing.

It is possible that the phenotypes seen after Septoria infection could be due to off target silencing. As *TaU4* is a small transcript that is made up of the highly conserved UBC fold, the similarity of each of the silencing fragments was first checked against the other four tested TaU proteins. None of the other four TaU

enzymes had nucleotide sequences with any significant similarity to *TaU4A* or *TaU4B* fragment.

A blast search of *TaU4* against *Triticum* species found that both *TaU4A* and *TaU4B* had over 100bp sequence identical to an unknown gene from wheat cv. Chinese Spring, chromosome 3B. However no more is known about this other gene. This points to the conclusion that TaU4 must have been silenced because of the change in phenotype and there being few other wheat genes with high similarity to either of the silencing fragments. Further investigations into the extent of silencing need to be performed to ascertain this fully.

Eighty five percent similarity between fragments is all that is needed for silencing to work on both genes, therefore potentially causing off target gene silencing. When the virus infects the cell it releases its RNA to be replicated by the viral encoded RNA polymerase. This then generates double stranded RNA that gets recognized by the plant enzyme DICER, which cleaves the double stranded RNA into small interference RNA's of around 21-23 nucleotides length. If one of these siRNA's is complimentary to an mRNA in the cell its gets broken down and expression is stopped. Therefore if even a small part of the fragment (>21bp) is compatible to another gene it can cause off target silencing and miss read phenotypes. It has been predicted that there could be up to 35% chance of miss silencing occurring in VIGS experiments (Xu et al 2006). Silencing efficiencies can also differ between fragments as it depends on: sequence of fragment (Thomas et al 2000), fragment position (therefore siRNA) in mRNA to be silenced (Birmingham et al 2006) and target mRNA secondary structure (Luo and Chang 2004).

If the silencing experiment were to be repeated there are other factors that would need to be taken into account to ensure that the silencing treatment could reach its full potential within the cell and therefore when measured would show a constant and strong silencing. This not only includes different silencing conditions but also ensuring that the leaves measured have the same extent of silencing as those that were infected with the Septoria fungus.

Another reason for the lower levels of silencing than expected could be due to its transient nature. As the virus replicates and undergoes recombination it has the potential to lose the fragment of the gene inserted. It is thought that the rate of loss is due to the size of the fragment inserted. Another factor affecting the levels of silencing may be the temperature in which the silenced plants are grown. In *A. thaliana* a protein has been identified, RdRP, that passes on the silencing signals to other cells, therefore priming the uninfected cells so that they can defend against the invading virus more effectively (Klahre et al 2002; Vaistij et al 2002). The plant defends against viruses in two different stages, the first stage relying on the infecting virus being present and the plant incorporating the siRNA from the virus into its silencing machinery. The second stage uses the previously incorporated siRNA as a template to make more copies (Ruiz et al 1998). RdRP functions in the secondary stage of plant defence against viruses. It is needed for the long distance movement of the signal between cells and continuation of the silencing after the viral template has gone (Vaistij et al 2002; Himber et al 2003).

Temperature is involved in the secondary silencing and has been found to be more effective at higher temperatures, with levels of viral siRNAs seriously reduced in low temperatures (Szittya et al 2003). Qu et al (2005) suggested that the lower levels of silencing at lower temperatures were due to a balance between the viral defense against silencing (by suppression of the plant silencing machinery) and the plants silencing defense being swayed in the viruses favour.

It would be interesting to see the levels of viral accumulation within the *N. benthamiana* to ensure that the fragments are being replicated and kept within the viral vector. The conditions for silencing are effective as the *PDS* gene has successfully been silenced (Figure 5), implying that it is either related to the cloning of the *TaU4A* and *TaU4B* into the *BSMV* vector or that the fragments are incompatible and lost easily from the *BSMV*. However when the wheat plants are infected with Septoria they are moved from 24^0C to 18^0C, which is the temperature that the Septoria favours for infection. This could stop the passage of the silencing signal to the new leaves grown in the 18^0C, which are also the leaves taken for the silencing check at 7, 14, 21 and 28dpi, but the leaves that are infected with Septoria would still be silenced. Also the stage of leaf collected

could have an effect, for instance in 0dpi silencing check the 3rd-5th leaf is collected and *TaU4* mRNA levels measured from this, then the leaves are trimmed for the Septoria infection and for later silencing checks the 5th-7th leaves are collected. A previous study using *BSMV* to silence *PDS* in wheat noted that the *PDS* photobleaching phenotype did not appear on the 5th leaf or newer, which could be due to the lack of silencing signal spreading to the younger leaves (Scofield et al 2005). It could, however, be due to unrelated issues such as RNA extraction, cDNA synthesis or real time PCR. Another study focusing on the silencing of *PDS* in wheat using *BSMV* viral vector also found a varied level in the silencing as well as the observation that the photobleaching phenotype did not cover the entire leaf but instead ran down the veins in the leaf. They suggest that this variation in silencing is due to differences in the leaf tissue area (Scofield et al 2005). This variation is at least partially in line with variation found in the silencing of *TaU4A* and *TaU4B* over the 28 days of infection, and coupled with the possibility that the younger leaves do not have the same levels of silencing could be suggesting a lower level of silencing when in fact the infected wheat leaves have been silenced.

6.3. Septoria ribosomal *60S L13* gene expression

The symptoms of Septoria infection develop at approximately 14 days and include the development of yellow necrotic tissue that turns brown. Eventually, towards 28 days of infection and high humidity, pcynidia are produced, which are like factories for growth of the spores. To ensure that the symptoms seen in the infected wheat were due to the Septoria fungus the expression levels of Septoria *60S L13* ribosomal protein were measured over the time course of the infected tissue. There was a gradual increase in the *60S L13* expression levels with larger increases between 14-16dpi, when the visible symptoms of the disease begin, and between 26-28dpi, when the Septoria prepares itself for spreading by spore production (Figure 9). To ensure that the levels of *60S L13* expression correspond with fungal biomass a DNA extraction should be done

and the levels of *60S L13* remeasured by real time PCR, however from this data we can imply that an increase in *60S L13* expression is due to an increase in Septoria fungal biomass. Measuring the *60S L13* gene amounts from extracted DNA can also be used in the future as an extra measurement of the amount of Septoria infection to further quantify the pycnidia and spore count data.

6.4. TaU4 protein

6.4.1. E2 activity

The expression of both *TaU4* and *TaU4*^{C88S} were both relatively high without any addition of IPTG to induce expression from the BL21 *E. coli* cells (Figure 12). However TaU4^{C88S} was more difficult to purify than the wild type protein. To increase the concentration of GST:TaU4^{C88S}, the purification can be done at 4^{O}C to avoid the breakdown of the GST from TaU4^{C88S} therefore having more protein for concentrating. Freeze thawing cycles can also cause protein breakdown, therefore if the protein was purified and concentrated on the same day this would avoid one cycle of freeze thawing. These methods would hopefully increase the concentration of the protein, which would lead to a more visible band on the E2 activating western blot.

The E2 ubiquitin conjugating ability of TaU4 was demonstrated in Figures 15 and 16, showing that ubiquitin can become bound to the active site cysteine of TaU4 in a similar manner to that of human UbcH5b, used as a positive control. The formation of TaU4-ubiquitin was shown to need all the components of the ubiquitination pathway, E1 enzyme, ATP and ubiquitin. The binding of the ubiquitin to the active site cysteine through a thioester bond was proven by subjecting the assay to reducing conditions, which caused a reduction in the ubiquitin bound to TaU4. It was also proven as the mutant of TaU4, which had the active site cysteine mutated to serine (which is a structurally similar but chemically different amino acid) could not bind to ubiquitin at all.

This proves that any changes in the phenotype in *TaU4A* and *TaU4B* silenced wheat is related to changes in the ubiquitination status of some proteins in wheat after Septoria infection. If the proteins could be extracted from non silenced, *TaU4A* and *TaU4B* silenced wheat that had been infected and not infected then any changes in the ubiquitinated protein profile could be assessed by western blotting with anti-ubiquitin (after separation by SDS PAGE gel electrophoresis).

6.4.2. TaU4 localization

Proteins up to 60kDa are generally accepted to be able to diffuse throughout the cell, into both the nucleus and the cytoplasm (Silver 1991). It has been suggested that proteins of 90kDa can also diffuse through the nuclear pore complex (Wang and Brattain 2007). TaU4 is a small protein of 17kDa that, based on size, could passively diffuse through the nuclear pore even with the YFP tag attached (YFP:TaU4 = 44kDa), which was used to visualize the cellular localization. This can therefore explain the cellular localization seen in Figure 17a, which is similar to that of YFP (but definitely due to YFP:TaU4 as seen in Figure 17c). Other E2 enzymes have been found to be distributed across the both the nucleus and the cytoplasm, for instance the human E2 enzyme UbcM2. Plafker et al (2004) studied UbcM2 localization and found that it changed depending on the ubiquitination status of UbcM2. When ubiquitin was attached to UbcM2 the E2 enzyme interacted with importin-11, a nuclear import protein, and so was imported into the nucleus. They also showed that without ubiquitin attached UbcM2 did not interact with importin-11 and stayed in the cytoplasm. This is slightly different to potential TaU4 passive diffusion into the nucleus as it has to be imported actively, using energy to transport the protein over the nuclear membrane at the nuclear pore complex (Richardson et al 1988). One way to test whether TaU4 localisation can be changed by ubiquitination would be to clone the $TaU4^{C88S}$ mutant (that was shown to be unable to become ubiquitinated in Figures 15 and 16) into *pEarleyGate104* and visualize the localization by confocal

microscopy. Other posttranscriptional modifications could also be influencing the cellular localization of TaU4, such as phosphorylation. S-receptor kinase (SRK) from *Brasscia* is known to be involved in rejection of self-pollen (self-incompatibility) at the plasma membrane of the stigma. After SRK recognizes self-pollen it phosphorylates ARC1 ubiquitin ligase, which then positively controls the self-compatibility response (Stone et al 2003). When Stone et al (2003) studied the localization of ARC1 they found that in the presence of a constitutively active SRK, lacking the transmembrane domain, the ARC1 localized from the cytosol to the ER membrane, probably at the 26S proteosome, and that this was dependent on the kinase activity of SRK. Using this example it could be possible that TaU4 enzyme is made in the cytoplasm and functions in the nucleus, but only localizes to the nucleus after it becomes phosphorylated. This may involve other interacting proteins that recognize the phosphorylation and then transport TaU4 into the nucleus. Using NetPhos 2.0 there is however no predicted phosphorylation sites on TaU4.

Potential targets for TaU4 within the nucleus include histones. The ubiquitination of histones has previously been indicated for the control of development, such as flowering times. Loss of function mutants of the E2 enzymes AtUbc1 and AtUbc2 from *A. thaliana* have an early flowering phenotype and smaller rosette leaves. Xu et al (2009) suggest that AtUbc1 and AtUbc2 help to mono-ubiquitinate the histone H2B and therefore activate repressors of floral genes.

To work out the targets of TaU4, the E3 ligase partner would need to be discovered first. Studying which family of E3 ligases the TaU4 interacts with could first be used to narrow down the interacting partners from the 1,300 E3 ligases found in plants. By using yeast two hybrid experiments with TaU4 and different E3 domains (HECT, RING etc) it may be possible to find potential E3 ligase partners for TaU4.

6.5. TaU1, TaU2, TaU3 and TaU5

6.5.1. Gene expression levels after Septoria infection

The level of each of the four genes expression in infected versus healthy tissue were tested in tissue 14 days post infection. This is only a rough guide to the gene expression levels as they can change throughout the dynamic infection process, as seen with *TaU4* (Figure 4). Each of the wheat E2 enzymes shows a change in expression level between the healthy and infected wheat at 14dpi. As plants do not have any way to escape the pathogens they have to have a wide variety of responses to pathogens. One way to control the proteins in the cell is to modify them and their interactions with other proteins using small attachable tags such as ubiquitin. Ubiquitin attachments can also target the proteins for degradation therefore changing the levels of proteins within the cytosol. One potential experiment that can come from this preliminary study of *TaU1*, *TaU2*, *TaU3* and *TaU5* is to check for any potential redundancy between the wheat E2 enzymes. It is also possible that two of the E2 enzymes could work together to ubiquitinate the target protein. For instance one may be needed for initiation of the ubiquitin chain and another could be needed for elongation. At least four ubiquitin molecules are needed to target proteins to the 26S proteosome. *TaU* genes with the same pattern would be coupled together for silencing to test any partnerships between the E2 enzymes, for instance TaU1 and TaU4 and TaU3 and TaU5 as these showed similar results.

6.5.2. Spore and pycnidia counts

The numbers for both the pycnidia count and the spore count are lower than expected possibly due to the leaves not being left in high humidity for long enough, therefore not allowing the full growth of the pycnidia and the spores. The Septoria infection phenotype shows that the Septoria has infected the leaves,

and appears to be more severe than when compared to the *BSMV:00* infection in the TaU4 experiments (Figure 7), suggesting that it is infact a lack of humidity that has meant that the pcynidia and spore counts were lowered than expected.

Both the pcynidia and spore counts for the Mock silenced wheat were lower than for the *BSMV:00* silenced wheat. A student t test showed that the pycnidia count for *BSMV:00* and Mock silenced wheat is not statistically different however the spore count showed that there was a statistically significant difference between the two of $p<0.05$. This experiment has only 6-9 technical replicates for each pycnidia count and 4 technical replicates for each spore count, which could lead to a lack of conclusive results.

6.5.3. *TaU3*

The study of *TaU3* shows promising results. There is an increase in expression at 14dpi after Septoria infection, after silencing there is a delay in the onset of visible symptoms and an increase in both pycnidia and spore count. One possible role of TaU3 is in activation of defence proteins against Septoria, which would explain the high expression at 14dpi in infected wheat and the increase in the pcynidia and spore count in *TaU3* silenced wheat.

The Septoria disease symptoms are possibly delayed in silenced wheat due to either the fungus not needing to switch to necrotrophic growth in a decreased defence environment or the Septoria needing TaU3, or its targets, to switch between biotrophic to necrotrophic growth. The first proposal assumes that the cue for the switch between biotrophic to nectrophic growth is similar to that of *C. graminicola* (a hemitroph that infects maize). To avoid the increased plant defence response the fungus switches to necrotrophic growth, therefore if TaU3 is involved in the activation of defence then when it is silenced there is a decrease in the defence. This allows the Septoria to continue to grow in the biotrophic phase, slowly spreading throughout the leaf tissue maximizing infection before switching to necrotrophic growth for reproduction.

A second proposal is that the Septoria needs TaU3 or its targets for the switch to necrotrophic growth. Pathogens hijacking plant ubiquitin machinery has been seen in bacteria infecting plants. The bacteria, such as *Agrobacterium tumefacians*, do not use SCF E3 enzymes to control cellular processes however they do encode for F-box proteins (the subunit of the SCF E3 enzyme complex that confers the specificity for the substrate to be ubiquitinated) using them in conjugation with plant encoded SCF E3 complex to ubiquitinate plant proteins and enhance infection (Tzfira et al 2004).

6.5.4. TaU2 and potential off target silencing

The primer set for TaU2B was rejected as it produced multiple amplified DNA fragments when tested. The primer set amplified a region within silencing fragment *TaU2B*, this therefore means that if primers in the silencing fragment can amplify multiple DNA genes, then siRNA produced from *TaU2B* fragment by DICER could not only silence the *TaU2* gene but also another gene, giving misleading results. Both the forward and reverse primer (of 20 base pairs, a similar size to siRNA produced in silencing) have bound to a gene that is not *TaU2*, implying there is a definite possibility of silencing a second gene. This could explain why there is less silencing for *TaU2* in wheat silenced with *TaU2B* fragment as the RISC complex is silencing multiple mRNA's therefore potentially focusing only half its resources on silencing *TaU2*. And if the other gene were expressed in higher concentrations then this other unidentified gene would potentially involve more of the RISC complexes in break down, leaving less to breakdown *TaU2* mRNA. This could also be masking any of the phenotypes seen in *TaU2B* silenced wheat, such as the increase in spore production seen. Another silencing fragment for *TaU2* would need to be tested that did not cross the nucleotides of the gene that are amplified by 2B primer.

6.6. BSMV effects on wheat

The experiments silencing *TaU1*, *TaU2*, *TaU3* and *TaU5* also show the difference that BSMV virus has on wheat defences, when *BSMV:00* and Mock silenced wheat are compared it appears that the viral infection causes wheat to be more susceptible to Septoria infection (Figures 21, 22 and 23). This can be seen by differences in the expression of *TaU2* and *TaU5* between *BSMV:00* and Mock silenced wheat when measured with 2A, 5A and a slight difference with 5B primers. *TaU2* expression is decreased and *TaU5* expression is increased after infection of the BSMV virus (Figure 20). The pcynidia and spore count also show a marked difference, with a significant increase ($p<0.05$) in the amount of spores produced in wheat silenced with *BSMV:00* (Figures 22 and 23) compared to Mock silenced wheat.

The BSMV vector has been modified with the coat protein removed and the fragment of the gene to silence cloned into the *γb* gene, although it is not known if the virus can still cause an infection within the cell. If the cell still recognizes foreign viral DNA and silences it then it may still turn on other viral defence mechanisms, which could include changes in *TaU1-5* expression. It was noted that viral symptoms could be seen in infected wheat after silencing treatment (data not shown). By silencing the genes using a virus vector this could the increase in gene expression (as the plant tries to defend itself against the virus) before the silencing can take effect. More viruses would lead to the more defence gene expression and more silencing. However this could lead to a positive loop, the plant senses a viral infection therefore turning on its silencing machinery and other defence mechanisms, the silencing takes effect, reducing the other potential defence protein (e.g. TaU5) causing the plant to increase expression of said defence protein.

TaU5 expression is higher in the *BSMV:00* than in Mock, *BSMV:TaU5A* and *BSMV:TaU5B* silenced wheat when measured with 5A primers. There is a large reduction in mRNA after silencing with *BSMV:TaU5A* and *BSMV:5B* when compared to *BSMV:00*, however not as much when compared to Mock silenced

wheat (with *BSMV:TaU5A* silenced wheat having more *TaU5* mRNA expression than Mock). There is also a large increase in *TaU5* expression between Mock and *BSMV:00*. This data indicates that when BSMV infects wheat it causes the increased expression of *TaU5* (Figure 20d). This is also shown in Figure 20e, measuring *TaU5* expression with 5B primers, with *BSMV:00* silenced wheat having a higher expression of *TaU5* compared to Mock silenced wheat.

TaU3 expression also increases after BSMV infection, as seen in Figure 20c between Mock and *BSMV:00* silenced wheat. The increase in expression after BSMV infection could therefore be masking any silencing when comparing mRNA levels with Mock infected wheat.

6.7. Conclusion and future work

VIGS is a useful tool in reverse genetics and can be used to assess gene function in Septoria-wheat interactions, however some extra controls still need to be assessed to ensure that off targeted silencing and full silencing is achieved. From the results it is predicted that TaU4 E2 enzyme acts as a repressor of positive defence regulators because when *TaU4* is silenced it causes a delay in Septoria disease symptoms and decrease in pcynidia and spore production in comparison to *BSMV:00* silenced wheat. In the future studying the expression levels and protein levels of known positive defence regulators along with *TaU4* silencing could help to confirm this prediction. Silencing of *TaU4* is not definitely confirmed but this may be due to experimental design and the ability of the silencing signal to spread to new leaves. This has to be investigated further under different silencing conditions. However due to the phenotype seen (Septoria infection, pcynidia and spore count) it appears that there has been silencing and that it is the measurement of the silencing that is causing the apparent low levels of silencing. Potentially due to TaU4's small molecular weight it is localized throughout the cytoplasm and nucleus that could be related to it's functioning in the nucleus, but only after it has been activated by a posttranscriptional modification such as phosphorylation. As well TaU4 there

are four other wheat E2 enzymes that may be involved in wheat defence against Septoria, TaU1, TaU2, TaU3 and TaU5. Silencing of two or more of the *TaU* genes together could show any potential working pairs of the E2 enzymes, for instance if one were to initiate the ubiquitin chain and another was involved in chain elongation. Further silencing and Septoria infection experiments need to be performed to strengthen the data found in this study. This could then be used together with the information revealed for TaU4 to generate potential interactions between the wheat E2 enzymes.

7. References

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8. Appendix

Pycnidia counts:

	Mean	SD	T test
BSMV:00	45.90	17.80	
BSMV:TaU4A	11.47	6.00	1.38E-04
BSMV:TaU4B	24.10	12.75	3.94E-03

	Mean	SD	T test BSMV:00	T test Mock
BSMV:00	30.88	23.31		
Mock	20.67	13.76		
BSMV:TaU1A	28.78	6.67	0.81	0.22
BSMV:TaU1B	27.14	11.82	0.70	0.39

	Mean	SD	T test BSMV:00	T test Mock
BSMV:00	30.88	23.31		
Mock	20.67	13.76		
BSMV:TaU2A	29.89	9.25	0.24	0.19
BSMV:TaU2B	24.13	9.96	0.47	0.62

	Mean	SD	T test BSMV:00	T test Mock
BSMV:00	30.88	23.31		
Mock	20.67	13.76		
BSMV:TaU3B	31.83	11.82	0.92	0.16

	Mean	SD	T test BSMV:00	T test Mock
BSMV:00	30.88	23.31		
Mock	20.67	13.76		
BSMV:TaU5A	32.00	10.38	0.90	0.13
BSMV:TaU5B	57.57	25.37	0.06	0.01

The mean pycnidia counts taken from counting a 2cm length of leave from Septoria infected wheat. The standard deviation (SD) was then worked out and a student t test performed.

Spore counts:

	Mean	SD	Ttest
BSMV:00	132.79	116.41	
BSMV:TaU4A	27.96	15.30	0.01
BSMV:TaU4B	78.92	43.78	0.12

	Mean	SD	T test BSMV:00	T test Mock
BSMV:00	11.75	6.24		
Mock	1.00	1.41		
BSMV:TaU1A	19.63	5.73	0.08	1.64E-05
BSMV:TaU1B	10.25	9.25	0.80	0.14

	Mean	SD	T test BSMV:00	T test Mock
BSMV:00	11.75	6.24		
Mock	1.00	1.41		
BSMV:TaU2A	3.75	1.50	0.08	0.04
BSMV:TaU2B	15.00	4.24	0.43	4.45E-03

	Mean	SD	T test BSMV:00	T test Mock
BSMV:00	11.75	6.24		
Mock	1.00	1.41		
BSMV:TaU3B	22.25	10.24	0.14	0.02

	Mean	SD	T test BSMV:00	T test Mock
BSMV:00	11.75	6.24		
Mock	1.00	1.41		
BSMV:TaU5A	9.00	4.76	0.51	0.04
BSMV:TaU5B	26.75	4.11	0.01	4.48E-04

The mean spore counts from Septoria infected wheat. The standard deviation (SD) was then worked out and a student t test performed.

TaU sequences:

TaU1
GGGAAAAGGAGCGAGGGCCTCTCTCTCCATCCCGACCTCTCGTGGCCGCCGCGAAGAA
AAGGAGCTTATC**ATG**GCTTCAAAACGTATCCTGAAGGAACTGAAGGACTTGCAGAAA
GATCCTCCGACATCATGCAGTGCAGGTCCTTCTGGTGAGGATATGTTCCATTGGCAG
GCAACCATTATGGGTCCTCCTGATAGTCCCTATGCTGGAGGTGTTTTCTTAGTGAAT
ATCCATTTCCCCCCGGACTACCCCTTCAAGCCGCCGAAGGTATCGTTCAAGACAAAGG
TCTTCCATCCGAACATCAATAGCAATGGAAGCATATGCCTTGACATTCTGAAGGAGC
AATGGAGTCCTGCTTTGACAATCTCTAAGGTTCTGCTTTCAATCTGCTCGCTGCTTAC
CGACCCTAACCCGGACGACCCTCTCGTCCCCGAGATTGCCCACATGTACAAGACGGAT
CGGTCCAAGTATGAGACGACAGCCCGCAGCTGGACGCAGAAGTATGCCATGGGA**TGA**
TACGAAGCCCATGCTCGTATCCACTGCTTGCATGCAGAAGATTGTGTCACTGTCCCGA
GAAACTATCTCTGGGCCATTATTTTCTTTGATTACTTGTCGGTCGCGTGTGTCTCCCT
GATCCATGTAGGATCGCGTCGTAGTCAAAACATCAACCTATCATTGCCAAAGTCCAT
AATGAAATTGACATATGCTTCTCGTTATAACTACGGTAGTGTTGGTTCTCCCAATAA
AAAAAAAAAAAAAACGA

TaU2
ATGGCATCAAAGCGCATCCTCAAGGAACTCAAGGACCTGCAGAAGGACCCGCCCACA
TCATGCAGTGCAGGTCCTGCTGGTGAGGACATGTTTCATTGGCAAGCAACAATTATG
GGACCCCCTGACGGTCCCTATGCCGGCGGTGTTTTCTTAGTGAACATTCATTTCCCTC
CGGATTACCCCTTCAAGCCACCAAAGGTATCTTTTAAGACAAAGGTCTTCCATCCTAA
TATCAACAGCAATGGAAGCATATGCCTTGATATTCTTAAGGAGCAGTGGAGCCCTGC
TTTGACGATCTCTAAGGTCTTGCTCTCTATCTGTTCCCTGCTGACCGATCCCAACCCG
GATGATCCCCTTGTTCCCGAGATTGCCCACATGTACAAGACGGACCGGTCAAAGTAT
GAGACGACAGCCCGCAGCTGGACGCAGAAGTACGCCATGGGT**TGA**

TaU3
GAGTTTCATCCCATTATTGGTTCTTGCGAGGAGGGGGGCGGAGAAGCGGAAGCAGGAGGC
AGAGAGGAGATCAGGCCAGAGGAGGGCGAGGGAGGGCGCGATGGCGTCCAAGAGGATAC
AGAAGGAGCTCAAGGATCTGCAGAAGGATCCCCCCCACCTCATGCAGCGCAGGCCCTGTGG
GTGAAGAT**ATG**TTCCATTGGCAGGCAACAATAATGGGCCCATCTGACAGCCCATATTCCGG
TGGAGTTTTCCTAGTTACTATCCACTTCCCTCCTGATTATCCTTTCAAACCACCAAAGGTGG
CATTCCGCACCAAGGTGTTCCATCCAAACATCAACAGCAACGGGAGCATTTGTCTGGACATC
CTCAAGGACCAATGGAGCCCCGCTCTGACCATTTCCAAGGTGCTGCTGTCCATCTGCTCCC
TGCTGTGTGACCCAAACCCTGACGATCCTCTGGTTCCTGAGATCGCTCACATGTACAAGAC
GGACCGGCACAAGTATGAGAGCACCGCCAGGACCTGGACGCAAAGGTATGCCATG**TAA**TG
TAAGACGGCGCACGGATCGCTGGAGTCAGTCTTGTTTGTAATATTCTCGATCTGTTCGTGT
GCCCGCCCGGTGTATGTGTCTGTCAAACTGGCACTCGTTGCTCAACTGTGTCACTTGGCGC
AGTGACTTGGAACATGGAACCGGTGTTGCATATTTTGGATTCAAAAAAAAAAAAAAACGA

TaU4
GATCCCCGACCCTCCCTTCCATGCCCAGGCCGACCTAAAGCCGTCCATTCCGTCGTCTCCAT
GCTCTAGGACAACGAGCACGCGGAGCCCACGTCTCCACCCCCACCGCCGGCCGCATCTCAG
AGAATTTCGAGG**ATG**TCGACTCCTTCAAGGAAGAGGCTGATGAGGGACTTCAAGCGGCTG
ATGCAGGACCCTCCTGCGGGCATAAGCGGGGCGCCGCAGGACAACAACATAATGCTGTGG
AATGCTGTGATTTTTGGCCCTGACGATAGCCCGTGGGATGGAGGCACGTTTAAGCTGACTC
TCCAGTTTAATGAAGAATATCCTAATAAGCCACCAACAGTTCGGTTTATTTCTCGGATGTTT
CACCCTAACATTTATGCTGATGGAAGCATATGCTTAGATATTCTACAGAATCAGTGGAGCCC
AATATATGATGTAGCTGCTATACTTACATCTATCCAGTCGCTGCTGTGTGATCCTAACCCAA

ATTCGCCTGCTAACTCAGAAGCTGCCCGCATGTTCAGTGAGAACAAGCGAGAGTACAACCG
CAAAGTGCGGGAGATTGTTGAGCAGAGCTGGACGGCAGACTAATAAGTTGAGCTCACCAT
GTGTTATCATGCGGTTTCTGTACCAAAATGTTTGTAACTAAAATGACTGAACTCTGTCGTTA
CCACCTGAAACAGCAACTTGCTTTGTTGCATCGTTTGGCTGGACGGTTGGAGATTGCTCTG
TCATGGCCTGTGTTTACACTGTCTGTATCTGGAACGCAAACATTTTGTAACGTCTTGTGTGA
GTTTGTAATGTTGTATCATATCACATTGATTCTGAAAAAAAAAAAAAAAACGA

TaU5
GGCTTTTCTCGCCTCCCTGTTCCCCGCCTTCTCTCTCTGCGGCAGCAATAAAAATAAATAAA
TCTCGCGCAGGTGCTGGGAGGAGGACGACGCACGCACCGTCACAAGCAGCTCAATCTCTTT
CTCCTTTTCTTTTCTCTGCCTCCTCCCTTATCCCCACGCCCAGGAAACGCGTCTCCGCAGGA
GGAAGGGAAGATTTCATCTTTTTTGCCCCCAAGATGTCCTCCCCAAGCAAGCGCCGCGAGA
TGGACCTCATGAAGCTGATGATGAGTGACTATAAGGTGGAGATGGTGAACGATGGGATGC
AAGAATTCTTCGTGGAATTCCGAGGGCCTACTGAAAGTATTTATCAAGGTGGTGTCTGGAA
GGTTAGAGTAGAACTGCCTGATGCATATCCTTACAAATCTCCGTCAATTGGGTTCATTAATA
AGATTTATCACCCAAATGTGGATGAAATGTCTGGTTCCGTATGTTTAGATGTTATCAACCAG
ACATGGAGCCCAATGTTTGATCTAGTAAATGTGTTCGAAGTCTTCCTTCCACAACTTCTGTT
GTACCCAAATCCGTCTGATCCATTAAATGGAGAGGCTGCTGCACTTATGATGCGAGATCGC
CCTGCTTATGAACAAAAAGTGAAAGAATTTTGTGAAAAATATGTGAAACCAGAGGATGCTG
GCATAACCCCAGAAGACAAGTCCAGTGATGAAGAGGAGCTTAGCGACGAAGATGACTCCGG
CGATGAGGATATAGTGGGCAAACCAGATCCTTAGTCACTACCACAACCCGTTGTATACAAT
GTTGGTTGCAAAATTTGCAATCAAATTGATCCAGAAAATCTGGGAAAGAGAACTTTGTATGT
CATGTAAATAAGTGTACTTGTATCTTATCCAATGTTGTTTTCTTCAGTTGACCAAAAAAAAAA
CAAAAACGA

Grey highlighted = fragment, underlined = real time primer amplified area, black highlighted area = start codon and end codon

Primer name	Sequence	Fragment size (base pairs)
18 S F (wheat)	CGGCTACCACATCCAAGGAA	200
18 S R (wheat)	GCTGGAATTACCGCGGCT	
TaU1 F	CACCATGGCTTCAAAACGTATCCTG	447
TaU1 R	TCATCCCATGGCATACTTCAGCG	
TaU1A F	AAGGAAGTTTAAGCGAAGAAAAGGAGCTTATCATGGC	200
TaU1A R	AACCACCACCACCGTGTAGTCCGGGGGGAAATGGATATTC	
TaU1B F	AAGGAAGTTTAAGCATATGCCTTGACATTCTGAAGG	200
TaU1B R	AACCACCACCACCGTTCATCCCATGGCATACTTCTGCG	
TaU2 F	CACCATGGCATCAAAGCGCATCCTC	447
TaU2 R	TCAACCCATGGCGTACTTCTGCG	
TaU2A F	*AAGGAAGTTTAA*ATGGCATCAAAGCGCATCCTC	200
TaU2A R	AACCACCACCACCGTACCTTTGGTGGCTTGAAGGGG	
TaU2B F	*AAGGAAGTTTAA*GCATATGCCTTGATATTCTTAAGG	200
TaU2B R	AACCACCACCACCGTTCAACCCATGGCGTACTTCTGCG	
TaU3 F	CACC**ATG**TTCCATTGGCAGGCAACAATAATG	357
TaU3 R	TTACATGGCATACCTTTGCGTC	
TaU3A F	*AAGGAAGTTTAA*AGCGGAAGCAGGAGGCAGAGAG	200
TaU3A R	AACCACCACCACCGTCCACCGGAATATGGGCTGTCAG	
TaU3B F	*AAGGAAGTTTAA*GGAGCATTTGTCTGGACATCC	200
TaU3B R	AACCACCACCACCGTTTACATGGCATACCTTTGCGTC	
TaU4 F	CACC**ATG**TCGACTCCTTCAAGGAAGAG	459
TaU4 R	TTAGTCTGCCGTCCAGCTCTGC	
TaU4A F	*AAGGAAGTTTAA*CGCCCAGGCCGACCTAAAGCC	200
TaU4A R	AACCACCACCACCGTTGCGGCGCCCCGCTTATGCC	
TaU4B F	*AAGGAAGTTTAA*TTTCTCGGATGTTTCACCCTAAC	200
TaU4B R	AACCACCACCACCGTGCGGTTGTACTCTCGCTTGTTC	
TaU5 F	CACC**ATG**TCCTCCCCAAGCAAGCGC	552
TaU5 R	CTAAGGATCTGGTTTGCCCAC	
TaU5A F	*AAGGAAGTTTAA*AGGAGGAAGGGAAGATTTCATC	200
TaU5A R	AACCACCACCACCGTGTTCTACTCTAACCTTCCAGAC	
TaU5B F	*AAGGAAGTTTAA*GTACCCAAATCCGTCTGATCC	200
TaU5B R	AACCACCACCACCGTCCACTATATCCTCATCGCCGG	
TaU4 serine F	GGAAGCATAAGCTTAGATATT	459
TaU4 serine R	AATATCTAAGCTTATGCTTCC	
PDS F	TCAGTCTTTGGGTGGTGAGGT	556
PDS R	AGGTTCGCAGTTCGGGAC	
Real time:		
TaU1A F	GCAGAAAGATCCTCCGACATCA	109
TaU1A R	AACACCTCCAGCATAGGGAC	
TaU1B F	GCTGCTTACCGACCCTAACC	93
TaU1B R	CGGGCTGTCGTCTCATACTT	
TaU2A F	AATTATGGGACCCCCTGACG	85
TaU2A R	TGGCTTGAAGGGGTAATCCG	
TaU2B F	TTGTTCCCGAGATTGCCCAC	86
TaU2B R	CATGGCGTACTTCTGCGTCC	
TaU3A F	CGATGGCGTCCAAGAGGATA	120
TaU3A R	GCCCATTATTGTTGCCTGCC	
TaU3B F	GTGACCCAAACCCTGACGAT	109
TaU3B R	ATGGCATACCTTTGCGTCCA	
TaU4A F	GGAGGCACGTTTAAGCTGAC	70
TaU4A R	TAAACCGAACTGTTGGTGGC	
TaU4B F	CGCTGCTGTGTGATCCTAAC	93
TaU4B R	GCACTTTGCGGTTGTACTCT	
TaU5A F	AAGATGTCCTCCCCAAGCAA	89
TaU5A R	ATCCCATCGTTCACCATCTCC	
TaU5B F	CGAGATCGCCCTGCTTATGAA	119
TaU5B R	TCGTCGCTAAGCTCCTCTTC	
Ubq F	TTGACAACGTGAAGGCGAAG	118
Ubq R	TGGATGTTGTAGTCCGCCAAG	
PDS F	GTCCCGAACTGCGAACCT	92
PDS R	GCCAGGTATTTCTGCTTTGTGT	

Buffers

Lysogeny broth (LB) Agar 1L

20g LB

15g Agar

up to 1L with deionised water

Autoclaved.

(Store at room temperature)

Super optimal broth (SOB) + $MgCl_2$ 1L

20g tryptone (2%)

5g yeast extract (0.5%)

0.5g NaCl (10mM)

0.19g KCl (2.5mM)

2.03g $MgCl_2$ (20mM)

up to 1L with deionised water

Autoclaved

(Store at room temperature)

SOC 1L

1L SOB (autoclaved)

4ml of glucose (filtered)

(Store at -20^{O}C)

4x SDS loading buffer 10ml

2ml 1M Tris base pH 6.8 (50mM)

4ml 100% glycerol (10%)

1ml 0.5M EDTA (12.5mM)

2.6ml deionised water

mix by inversion

0.8g SDS (2%)

mix with loop gently

0.4ml 14.7M β-mercaptoethanol (1%)

8.0mg bromophenol blue

(Store at -20^{o}C)

YPD 1L

20g bacto peptone

10g yeast extract

20g dextrose

15g agar

up to 1L with deionised water

autoclaved.

(Store at room temperature)

TE buffer 1L

10ml 1M Tris.HCl pH 8.0 (10mM)

2ml 0.5M EDTA pH 8.0 (1mM)

988ml deionised water

(Store at room temperature)

SDS PAGE 10x running buffer 1L

30.35g Tris base (250mM)

141.75g glycine (1.89M)

10g sodium dodecyl sulfate (35mM)

up to 1L with deionised water

pH should be 8.3 with no adjustment

(Store at room temperature)

SDS PAGE 1x running buffer 1L

100ml SDS PAGE 10x running buffer

900ml deionised water

(Store at room temperature)

SDS PAGE 10x transfer buffer 1L

30.2g Tris base (250mM)

144g glycine (1.92M)

up to 1L deionised water

(Store at room temperature)

SDS PAGE 1x transfer buffer 1L

100ml 10x SDS PAGE 10x transfer buffer

200ml methanol

700ml deionised water

(Store at $4^{O}C$)

10x TBS 1L

24.3g Tris base (200mM)

80.06g NaCl (1.37M)

800ml deionised water

adjust pH to 7.6 using HCl

up to 1L with deionised water

(Store at room temperature)

1x TBST 1L

100ml 10x TBS

899ml deionised water

1ml Tween 20 (1%)

(Store at room temperature)

1M HEPES 1L

238.3g HEPES (1M)

800ml deionised water

adjust pH to 8.0 using NaOH

up to 1L with deionised water

(Store at room temperature)

GSTrap 4B binding buffer 1L

8.18g NaCl (140mM)

0.2g KCl (2.7mM)

1.42g Na_2HPO_4 (10mM)

0.24g KH_2PO_4 (1.8mM)

Adjust to pH 7.4 using HCl

Up to 1L with pure deionised water

Filter through a 0.45μM filter

(Store at $4^{O}C$)

GSTrap 4B elution buffer with glutathione 1L

6.06g Tris base (50mM)

6.15g Reduced glutathione (20mM)

Adjust to pH 8.0 using HCl

Up to 1L with pure deionised

Filtered through a 0.45μM filter

(Store at $4^{O}C$)

GSTrap 4B elution buffer without glutathione 1L

6.06g Tris base (50mM)

Adjust to pH 8.0 using HCl

Up to 1L with pure deionised water

Filtered through a 0.45μM filter

(Store at $4^{O}C$)

10x Ubiquitin assay buffer 1L

100ml of 1M HEPES (pH 7.5, 10mM)

58.44g NaCl (1M)

1.9g $MgCl_2$ (20mM)

4μl of 100mM ATP (400μM)

Up to 1L with pure deionised water

(Store at $-20^{O}C$)

2x non-reducing SDS loading buffer 1L

50ml of 1M Tris base (pH 7.0, 50mM)

50g Sodium dodecyl sulfate (5%)

200ml glycerol (20%)

20mg bromophenol blue (30μM)

Up to 1L with deionised water

(Store at room temperature)

Protein extraction buffer 100ml

0.88g NaCl (150mM)

1ml IPEGAL (1%)

0.5ml Sodium deoxycholate (0.5%)

0.1g Sodium dodecyl sulfate (0.1%)

5ml of 1M Tris base (pH 8.0, 50mM)

0.2ml of 0.5M EDTA (1mM)

10 Protease inhibitor tablets

Up to 1L with deionised water

(Store at -20^{O}C)

Coomassie stain 1L

1g Coomassie brilliant blue

500ml methanol (16M)

400ml deionised water

100ml glacial acetic acid (6M)

Filter through filter paper

(Store at room temperature)

Destain

500ml deionised water

400ml methanol (12.8M)

100ml glacial acetic acid (6M)

(Store at room temperature)

ECL 200ml

Solution A 100ml

1ml luminol solution (1%)

0.44ml coumaric acid solution (0.44%)

10ml Tris base 1M, pH 8.5 (0.1M)

Up to 100ml with deionised water

(Stored in the dark 4^{O}C)

Solution B 100ml

64µl 30% hydrogen peroxide (0.02%)

10ml Tris base 1M, pH 8.5 (0.1M)

Up to 1L with deionised water

(Stored at 4^{O}C)

Add solution A + B in equal volumes before use.