CHAPTER 3

Materials and methods

1. Barley plant material

Barley seed were obtained from the Small Grain Institute, Bethlehem, of the Agricultural Research Council (ARC-SGI). One cultivar was used, namely Puma 15, these plants were grown under greenhouse conditions and RNA was extracted by using the plant material surrounding the inoculation point. All the plants used for inoculation were in the third leaf stage. The cultivar was divided into two sections, the inoculated (tester) and control (driver). Only the tester was inoculated with *Fusarium graminearum* spores.

2. Fungal isolates

F. graminearum, obtained from the Plant Protection Research Institute (ARC-PPRI) were grown on carnation leaf agar (CLA, water agar with a carnation leaf) medium for two weeks and stored at room temperature. Spores were harvested by adding 5ml sterile water to the petri dish and scraping it with a glass pipette. 2ml of the spore suspension was transferred into eppendorf tubes and the spore concentration measured with a haemocytometer. The spore suspension was stored in 15% glycerol at -20℃.

3. Fungal infection

Three sets of barley plants at the three-leaf stage from thecultivar were inoculated with *F. graminearum* spores. 10µl of a spore suspension at a concentration of 10 750 000 spores/ml containing 1% Tween™ was placed in the sheath between two leaves. Plants that were inoculated were compared to uninoculated plants. Both inoculated (tester) and control (driver) plants were left for 24 hrs (Bernardo *et al*., 2007; Desmond *et al*., 2006) in glass chambers at a temperature of 18℃ and 100% relative humidity. When larger numbers of plants were inoculated, the relative humidity in the growth room was increased with a humidifier.

Barley plants from Puma 15 were infected with *F. graminearum* at the spikelets to confirm the susceptibility of the plant to the pathogen. The infected seeds were placed on potato dextrose agar (PDA) medium to see if the pathogen was indeed *F. graminearum.*

4. General techniques

4.1 Total RNA extractions

RNA was extracted from the barley plants with the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's specifications. In short, plant material (six repetitions of each sample) was harvested 24 hrs after inoculation. Approximately 100mg of plant material of the control (driver, supplemented with fungal material harvested from *in vitro* growth) and inoculated (tester) were used. The control and inoculated plant tissue were ground in liquid nitrogen to a fine powder and fungal mycelium was added to the control extractions. The samples were placed in 2ml eppendorf tubes with an extraction buffer, vortexed and incubated for 2 min at 56℃. The samples were then transferred to a QIAshredder spin column to break open the cells and centrifuged for 2 min at full speed. The supernatant was transferred to a new microcentrifuge tube and 96% ethanol was added. This was transferred to an RNeasy spin column and centrifuged to bind the sample to the column. The flow-through was discarded. A washing buffer was added to wash the sample and centrifuged. DNA was removed by on-column addition of DNase I (30 Kunitz units) (Qiagen) for 15 min. Then the columns were washed twice with washing buffer for 15 sec and a 2 min centrifugation to remove ethanol. The flow-through was discarded between the two centrifugation steps. The RNeasy spin column was transferred to a new tube and RNAse-free ddH_2O was added to elute the sample and stored at -80℃.

4.2 Agarose gel electrophoresis

Most gel electrophoreses were performed on 1% agarose gels run at 2 $V.cm^{-1}$ in 1x TAE (40mM Tris-acetate, 1mM EDTA) and stained with either ethidium bromide or Goldview (Sylvean Biotech) to determine the integrity of the samples. Visualisation was performed with a standard UV transilluminator.

4.3 Messenger RNA (mRNA) extraction from total RNA

The absorbance of the RNA control and inoculated samples was read at A_{260} and A_{280} using a spectrophotometer (Eppendorf). The mRNA fraction was purified by using the Oligotex mRNA Spin-column kit (Qiagen) following the manufacturer's specifications. In short, before extraction started, the Oligotex Suspension was heated to 37°C in a water bath. The dilution buffer was heated to 70°C. Approximately 15µg total RNA was used and placed in an RNase-free microcentrifuge tube. RNase-free water was used to fill up the volume to 250µl. 250µl Binding buffer and 15µl of the Oligotex suspension was added to the sample. The content was mixed by pipetting and incubated for 3 min at 70°C to disrupt the secondary structures of the RNA. The samples were removed and placed on a heating block at 25°C for 10 min. This allowed hybridisation between the Oligotex particles and poly-A tail of the mRNA. After this incubation, the samples were centrifuged for 2 min at full speed and the supernatant removed. The pellet was resuspended in 400µl Resuspension buffer, vortexed and pipetted into a small spin column (Qiagen) and centrifuged for 1 min at maximum speed. The spin column was transferred to a new 1.5ml microcentrifuge tube. Washing buffer was added and again centrifuged for 1 min at maximum speed. The flow-through was discarded and the spin column was transferred to a new microcentrifuge tube and 20µl of hot Elution buffer was pipetted onto the column and centrifuged for 1 min to elute the mRNA. This step was repeated with fresh heated elution buffer to increase the total yield of mRNA. The mRNA samples were immediately used to prepare cDNA.

4.4 Complementary DNA (cDNA) synthesis

The cDNA Synthesis System (Roche) was used for first and second strand cDNA synthesis.

4.4.1 First strand cDNA synthesis

A sterile 1.5ml microcentrifuge tube was used to pipette 19µl of mRNA and oligo dT_{15} primer (0.4mM) to a total volume of 21µl. This was incubated at 70°C for 10 min and placed on ice. The following components were added and mixed to a final concentration in 40µl: 1x RT-buffer; Dithiothreitol (DTT) (10mM); Avian Myeloblastosis Virus (AMV) reverse transcriptase (50U); RNAse inhibitor (25U); Deoxynucleotide triphosphate-Mix (dNTP) (1mM each). The reactions were incubated at 42°C for 60 min and then placed on ice.

4.4.2 Second strand cDNA synthesis

The following was added to the first strand synthesis reactions: 1x second strand buffer; dNTP-mix (0.1mM); second strand enzyme blend and redistilled water to a final volume of 150µl. This was incubated for 2 hrs at 16°C after which T4 DNA Polymerase (20U) was added and incubated for 5 min at 16°C.

4.5 cDNA purification

The MinElute Reaction Purification Kit (Qiagen) was used for cDNA purification and the manufacturer's specifications were followed. In short, 150µl of each sample was used and added to Binding buffer. The samples were mixed and placed on a MinElute column (Qiagen) to bind the cDNA and centrifuged. The flow-through was discarded

and Washing buffer was added to the columns and they were centrifuged at maximum speed. The flow-through was discarded and the columns were centrifuged to ensure that all ethanol in the samples were removed. The cDNA was eluted twice with 20µl elution buffer (10mM Tris-Cl, pH 8.5).

5. Suppression subtractive hybridisation (SSH)

The SSH was done using the PCR-Select™ cDNA Subtractive (BD Biosciences Clontech) kit following the manufacturer's instructions.

5.1 RsaI digest

38µl Double stranded cDNA was digested with RsaI in a 50µl reaction containing the following: 1x RsaI restriction buffer; RsaI (20U) and water were added to a final volume of 50µl. The samples were gently mixed and incubated over night at 37°C. The restriction digest was evaluated by agarose gel electrophoresis as stated previously. After digestion the samples were cleaned using the MinElute Reaction Purification Kit (Qiagen).

5.2 Southern Blot

A Southern Blot was performed to transfer the digested cDNA to a nylon membrane (Roche) through capillary action. This was done to test the efficiency of the samples. 0.4 M NaOH was used as the transfer buffer. The membrane was rinsed in 100mM Tris (pH7.4), cross-linked under UV light for 3 min and stored dry at room temperature (Sambrook and Russel, 2001).

5.3 Ligation

No adaptors were ligated onto the ends of the driver cDNA. The tester sample was divided into two and adaptors 1 and 2R ligated to the cDNA in the two pools respectively. A ligation mix with a total volume of 10µl was prepared containing 1x ligation buffer and 400U T4 DNA ligase. For the ligation reaction 2µl undiluted tester cDNA was used and Adaptor 1 (0.01mM) (5´-CTAATACGACTCACTATAGGGCTCGAG CGGCCGCCCGGGCAGGT-3´ 3´-GGCCCGTCCA-5´) and Adaptor 2R (0.01mM) (5´-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3´ 3´-GCCGGCTC CA-5´) respectively. This was incubated at 16℃ over night. 1µl of EDTA/Glycogen was added and the samples were heated at 72℃ for 5 min to inactivate the ligase. The ligation efficiency was tested with a Polymerase chain reaction (PCR) using PCR Primer 1 (0.01mM) (5´-CTAATACGACTCACTATAGGGC- 3´); dNTP mix (10mM); and 10x PCR reaction buffer (BD Bioscience Clontech). The thermal cycle conditions were for 1 cycle, 72℃ for 5 min; 94℃ for 60 sec followed by 94℃ for 30 sec; 65℃ for 30 sec; 72℃ for 2 min 30 sec for 30 cycles. A 1% agarose gel was run at 2 $V.cm^{-1}$. A Southern blot was done to transfer the PCR product to a membrane. Samples were stored at -20℃ and used as a back-up sample for future studies.

5.4 Hybridisation

5.4.1 First hybridisation

In the first hybridisation, a ratio volume of 150:1 of driver and tester cDNA (1.5µl each) was added together and heat denatured. 1x hybridisation buffer was mixed with cDNA in PCR tubes with 1.5µl Adaptor 1-ligated tester and Adaptor 2R-ligated tester respectively to a final volume of 4µl. This was overlayed with a drop of mineral oil. The samples were then denatured for 90 sec at 98℃ and incubated at 68℃ for 7 hrs.

5.4.2 Second hybridisation

1µl Fresh driver cDNA was mixed with 4x hybridisation buffer and water, overlayed with a drop of oil and denatured at 98℃ for 90 sec. The freshly denatured driver and two primary hybridisations were mixed together immediately and incubated over night at 68℃. After this incubation 200µl Dilution buffer was added and the reactions incubated at 68℃ for 7 min.

5.5 PCR amplification

The enriched cDNA hybridisation reaction was PCR amplified using a Biorad MyCycler. 1µl of the diluted hybridisations were used as template in the PCR reaction. 1x PCR buffer (BD Bioscience Clontech); dNTP mix (10mM); PCR Primer 1 (0.01mM); 1x BD Advantage cDNA Polymerase Mix (BD Bioscience Clontech) was used for the master mix and added to the samples. The reaction conditions were an initial incubation of 5 min at 75℃, followed by 25 sec at 94℃. This was followed by 27 cycles of 94℃ for 10 sec; 66℃ for 30 sec; and 72℃ for 90 sec. The PCR products were run on a 2% agarose gel at 2.5 $V.cm^{-1}$ as previously described and the samples were diluted 1:10 for use as template in the secondary PCR.

The secondary nested PCR amplification was performed to further enrich for differentially expressed genes. A total volume of 25µl was used for PCR reactions. 1x PCR buffer (BD Bioscience Clontech); Nested PCR primer 1 (0.01mM) (5´-TCGAGCGGCCGCCCGGGCAGGT-3´); Nested PCR primer 2R (0.01mM) (5´-AGCGTGGTCGCGGCCGAGGT-3´); dNTP mix (10mM); and 1x Advantage cDNA Polymerase Mix (BD Bioscience Clontech), adding 1µl of sample (1:10 dilution of the primary PCR products). The thermal cycle conditions were 94℃ for 10 sec; 68℃ for 30 sec; 72℃ for 90 sec for 35 cycles as no amplification products could be detected after 15 cycles as recommend by the kit. The samples were separated on a 2% gel. PCR

amplification products were cleaned with the MinElute Reaction Purification Kit (Qiagen) and stored at -20℃.

5.6 Random primed probe labelling

DNA labelling was done using the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche). All the positive samples from the PCR amplification were combined and 1µl of sample was used adding double distilled water to a volume of 16µl. This was denaturated at 98℃ for 10 min to separate double stranded (ds) cDNA. 4µl of DIG-High Prime was added and incubated over night at 37℃.

5.7 Determination of labelling efficiency

Labelling efficiency was calculated by comparing serial dilutions (as recommended by Roche) of labelled probe to the labelled control. 1µl of each dilution was spotted onto a nylon membrane, which was cross-linked through UV-light and detected according to the manufacturer's specifications. The membrane was then exposed to an x-ray film (Roche), which was developed according to standard procedures.

5.8 Determining efficiency of SSH with Southern Blot

Southern blot hybridisations were performed with DIG-Easy Hyb (Roche) and hybridisations (see previously used hybridisations) were performed over night at 37℃. Stringency washes were done with a stock solution of 10x Sodium citrate buffer (SSC) (300mM NaCl, 3000mM $Na_3C_6H_5O_7$). From that 2x SSC containing 0.1% Sodium dodecyl sulphate (SDS); and 0.5x SSC, 0.1% SDS were used for 5 min to remove the excess probe on the membrane. The hybridisations were then detected as stated above.

5.9 Stripping and re-probing of DNA blots

After completion of the Southern Blot, the membrane was stripped according to Roche's specifications and stored dry at room temperature.

6. Cloning of DNA into competent *Escherichia coli* cells

The secondary PCR products were ligated into pGEM-T Easy (Promega) according to the manufacturer's specifications. 1µl of each of the ligation reactions were used to transform 30µl of competent ElectroMAX™ DH10B™ *E. coli* cells (Invitrogen). The cells were transformed at 1.8kV using a Multiporator® electroporator (Eppendorf) as recommended by the manufacturer. After transformation 970µl SOC (SOB, 20mM glucose) (Invitrogen) was added and the cells were shaken for 60 min at 37°C. The cells were plated out on plates (Nunc™) containing LB medium (10g.l^{-1}Tryptone, 5g.l^{-1} Yeast extract, 15g.l^{-1} Agar, 10g.l^{-1} NaCl), ampicilin (0.05g.l^{-1}), Isopropylthio β-D galactoside (IPTG, 800mM) and 5-Bromo-4-chloro-3-indolyl-β-D-Galactopyranoside (X-Gal, 20mg/ml). Transformation efficiency was determined by α-complementation (Sambrook and Russel 2001).

6.1 Plasmid preparations

White colonies were transferred from the transformation plates to 96 DeepWell PP Plates (Nunc™) containing 1ml LB medium and ampicillin. The plates were incubated over night at 37°C and stored at 4°C while working from them. For long term storage 15% glycerol was added and the plates were stored at -20°C or -80°C.

6.2 Colony PCR

A colony PCR was done to amplify the DNA fragments cloned into the plasmid vectors, using a PCR reaction mixture. This contained 1x S-T ExSel PCR buffer, dNTP's (0.2mM each), Primer Sp6 (0.01mM) (5´-TATTTAGGTGACACTATAG-3´), Primer T7 (0.01mM) (5´-TAATACGACTCACTATAGGG-3´), 0.04U Taq (Southern Cross Biotech), and water to a final volume of 10µl. Sample from the 96 well plates was added using sterile toothpicks. The thermal cycle conditions were one cycle of 94℃ for 1 min; 94℃ for 30 sec; 50℃ for 30 sec; 72℃ for 1 min for 30 cycles; and a final elongation step at 72℃ for 5 min for 1 cycle.

6.3 Purification of PCR products in micro plates

The PCR products were cleaned through ethanol precipitation by addition of two volumes ethanol (96%) and 0.2 volumes sodium acetate (3M; pH 4.6). The samples were centrifuged for 10 sec at 2300rpm. This was incubated on ice for 1 hr and then centrifuged for 1 hr and 20 min. After centrifugation, the supernatant was removed. To dry the plates fully, it was covered with tissue paper and centrifuged upside down for 10 sec. The pellet was washed with 150 - 250µl 70% ethanol and centrifuged for 15 min. The plates were again dried and centrifuged for 10 sec. 70% ethanol was added again and centrifuged for 15 min. The supernatant was aspirated and the pellet air-dried at 60℃. The pellet was resuspended in 5µl ddH_2O.

6.4 Cycle sequence PCR

A cycle sequence was done to amplify the DNA fragments for sequencing. This mixture contained 1x sequencing buffer, Big Dye Terminator (Applied Biosystems) (0.5µl), Primer T7 (0.01mM) and water. 2µl of colony PCR sample was added to a final volume

of 10µl. The thermal cycle conditions were 96℃ for 10 sec; 50℃ for 5 sec; 60℃ for 4 min for 26 cycles with a final elongation step at 4℃ for 30 min.

6.5 Sequencing

The purified product was separated and detected on an ABI 3130 xl Genetic Analyser (Applied Biosystems) at the University of Johannesburg. DNA sequence editing was performed with Sequencher 4.6 and National Centre of Biotechnology Information (NCBI) database searches with BLASTn and BLAStx analyses. Cells harbouring plasmids containing fragments of interest were stored in 15% glycerol at -20℃.

7. Northern Blots

RNA was extracted at 24 hr time intervals (0 hpi - 120 hpi) and Northern blot analyses were done to study the expression of two of the identified TDF's. The extracted total RNA (500ng) were slot-blotted (Hoefer) onto a nylon membrane (Roche) through vacuum filtration according to standard protocols (Sambrook and Russel, 2001). These hybridisations were done using SSH10 and SSH11 as they shared homology to known sequences. Hybridisation and stringency washes were done as described in Southern Blot analyses. Stripping was done with 10mM Tris-HCl (pH 7.5) at 65℃ and 2x SSC containing 0.1% SDS for 5 min at 25℃. The x-rays were scanned and the density profiles were obtained for each expression profile using Scion Image (Scion Inc.). The expression analyses were confirmed with the use of biological replicates.