Chapter 4

Results

1. Total RNA extraction

Total RNA was extracted from the infected (tester) and uninfected (driver) barley (Puma 15). The final quantity of 15µg RNA was used for the SSH. The quality was measured by agarose gel electrophoresis (Figure 4.1) with the intact ribosomal RNA bands and smears visible. Messenger RNA was purified from the total RNA and second strand cDNA was synthesised for use in the SSH procedure.

2. Suppression subtractive hybridisation

2.1 Adaptor Ligation

To enrich for differentially expressed genes the RNA from *F. graminearum* infected as well as control barley plants were used in an SSH protocol. After cDNA synthesis the cDNA was digested with RsaI and two separate adaptors differentially ligated onto each end. The ligation efficiency was analysed by PCR amplification using adaptor specific primers (PCR Primer 1) (Figure 4.2). PCR products containing smears were taken as positive results for the ligation efficiency test. The smears produced fragment sizes between 80bp and 500bp.

2.2 Hybridisation reactions

During the SSH procedure two hybridisation reactions were performed. In the first hybridisation, each tester cDNA pool was separately hybridised to excess driver cDNA. This normalised the different cDNA samples. In the second hybridisation, the samples from the first hybridisation were mixed together and fresh driver cDNA was added to enrich for differentially expressed genes. These differentially expressed genes were PCR amplified (Figure 4.3). After the initial 15 cycles of PCR as recommended by the protocol, no product was visible and an additional 20 cycles were performed. The bands produced fragments of approximately 350bp – 500bp in

size. The PCR products were obtained from two individual primary PCR reactions and used as template for the secondary PCR's.

2.3 SSH efficiency through Southern Blot analysis

Efficiency of the SSH procedure was verified through Southern blot analysis (Figure 4.4). This indicated hybridisation to the tester cDNA (lane 2) and less hybridisation to the driver cDNA (Lane 3). The Southern blot had high background noise, but indicated with enough clarity that the SSH subtraction was successful.

2.4 Sequencing

The PCR products from the secondary PCR were cloned and sequenced. From the sequence analysis six clones were identified that contained different transcript derived fragments (TDF's). The sequences of these clones were compared to GenBank using the BLASTx (Table 4.1) and BLASTn (Table 4.2) algorithm. The majority of the identified TDFs were related to Rubisco and several displayed homology with stress related proteins. TDF alignments to their respective database hits are displayed in Figures 4.5 - 4.10.

Clone SSH10 showed strong homology at both amino acid (BAB40923, E= $2e^{-68}$) and nucleotide level (NM 001051729, E= $2e^{-138}$) to a putative selenium binding protein from *Oryza sativa* (*japonica* cultivar-group) with 98% and 91% homology respectively. Clone SSH11 shared significant amino acid (AAK49456, E= $4e^{-39}$) and nucleotide (AF307145, E= $1e^{-110}$) homology with a glutamine-dependent asparagine synthetase 1 protein from *Hordeum vulgare* subsp. *vulgare* (AAK49456) with 90% and 95% homology respectively.

Clone SSH12 and SSH15 shared no homology to any known protein hosted at GenBank. There was non-significant homology (E=4.4) to a hypothetical protein from *Theileria annulata* (XP_955549) with a homology of 36% for SSH12. At a nucleotide level SSH12 shared no significant homology. Clone SSH15 displayed a very low

amino acid (BAC16191, E= 0.18) and high nucleotide (AK248318, E= $2e^{-83}$) homology to a hypothetical protein from *Oryza sativa* (*japonica* cultivar-group) with 34% and 93% homology respectively. Clone SSH13 showed strong homology at both amino acid (AAA33396, E=$9e^{-44}$) and nucleotide (AK248764.1, $2e^{-153}$) level to a light harvesting chlorophyll a/b protein precursor from *Lemna gibba* with 97% homology and with a *Hordeum vulgare* subsp *vulgare* cDNA clone with 100% homology.

Clone SSH16 showed strong homology (E= $3e^{-15}$) to a hypothetical protein from *Vitis vinifera* (CAN65763) with a homology of 84%. Upon inspection of the homology at nucleotide level it was evident that it was from the 26S ribosomal gene of *V. vinifera* (AY049041.1) with a 97% homology.

3. Infection of barley plants

After spikelet infection of Puma 15 with *F. graminearum*, the seeds showed a decolourisation typical of FHB infection (Figure 4.11A). Figure 4.11B shows the characteristic red colony morphology of this fungus upon re-isolation from the infected heads.

4. Expression analyses

The Northern blot analysis of both clones displayed hybridisation to both the inoculated plants and the uninoculated control plants. Northern blot analysis of the putative selenium binding homologue (SSH10, Figure 4.12) showed low levels of induced gene expression in the inoculated plants as compared to the control at 24 hpi. Thereafter the inoculated and control samples displayed similar regulation profiles with a down-regulation at 96 hpi for both the inoculated and control samples (higher RNA concentrations were transferred to the membrane for time points 96 and 120). For the asparagine synthetase homologue (SSH11) there was a significant up-regulation in the inoculated sample at 24 hpi (Figure 4.12). This was followed by a

down-regulation by 48 hpi. At 72 hpi there was a significant up-regulation evident in both the control and the inoculated samples followed by steady state levels at 96 hpi and 120 hpi.

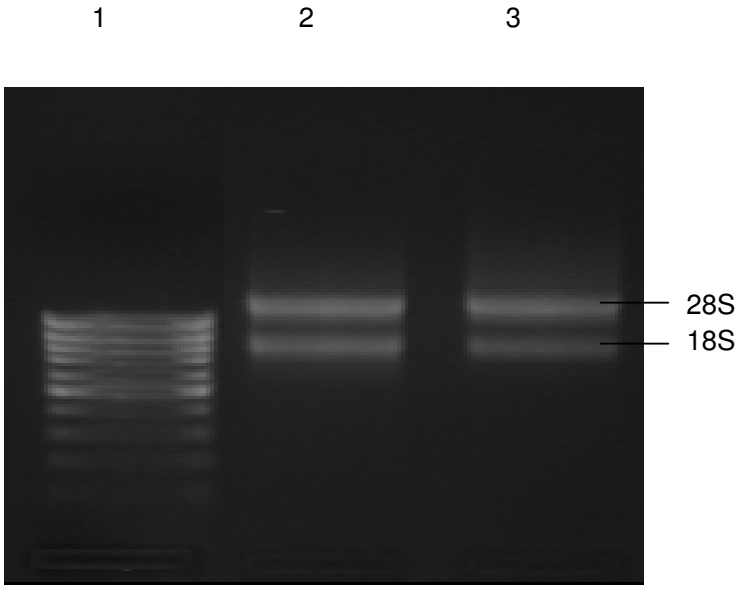

Figure 4.1: Total RNA extraction with a 1 kb ladder (lane 1), driver sample (Puma15) in lane 2 and tester sample (Puma15) in lane 3. In both samples the rRNA bands are clearly visible indicating that the RNA quality was high.

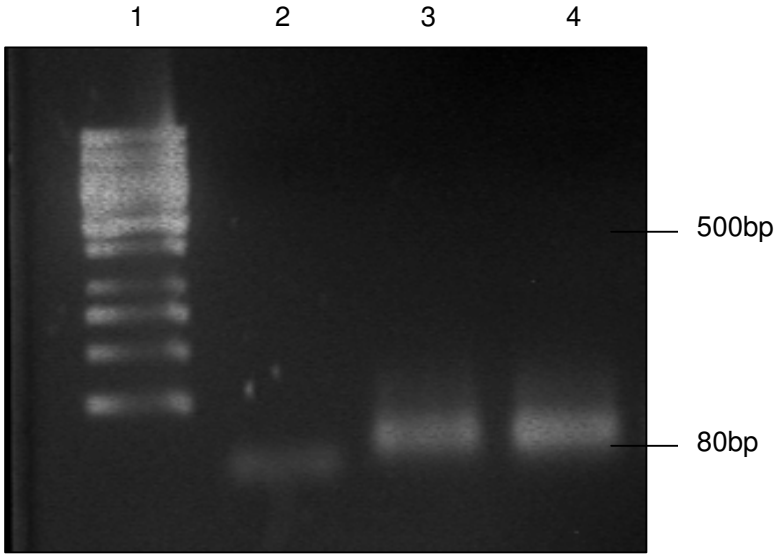

Figure 4.2: Ligation efficiency test of tester cDNA. PCR reactions were performed with PCR Primer1 following the primary PCR protocol. Lane 1 includes the 1 kb ladder, lane 2 contains the negative control with primer dimer formation, lanes 3 and 4 contain adaptor ligated tester samples with smears that range from 80 to 500bp. These smears are larger than the formed primer dimers and were taken as a positive indication of the ligation reactions.

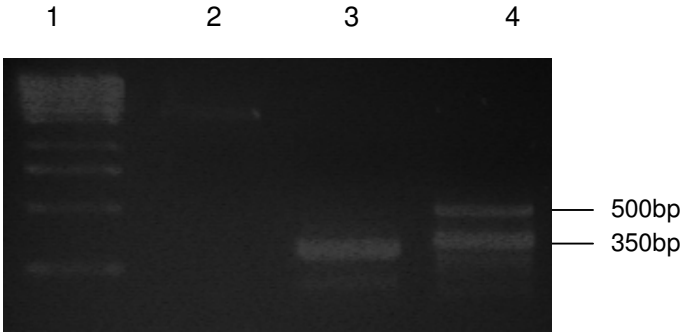

Figure 4.3: PCR products obtained after two rounds of PCR amplification with Nested PCR primer 1 and Nested PCR primer 2R. Lane 1 includes the marker (1kb), lane 2 contains the negative control and lanes 3 and 4 contain fragments amplified from the enriched pool obtained from the inoculated tester sample.

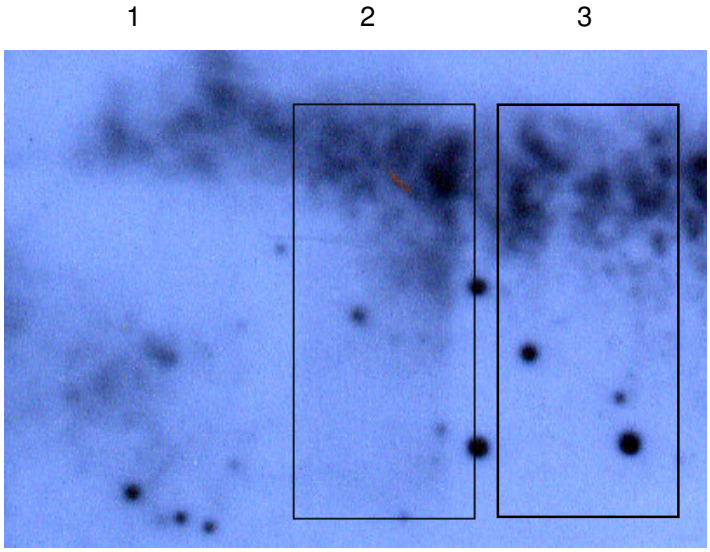

Figure 4.4: The determination of SSH efficiency through Southern Blot analysis, showing the hybridisation of sample 15. Lane 1 contains the marker, lane 2 contains the tester cDNA and lane 3 contains the driver cDNA. No hybridisation was expected to the marker in lane 1. The block highlights the difference in hybridisation levels between the subtracted tester and unsubtracted driver samples.

Table 4.1: Putative identities of the SSH fragments attained using the BLASTx algorithm (http://www.ncbi.nlm.nih.gov/blast).

Clone	*Closest homology (BLASTx)*	*Organism*	*Significance*	*Homology %*
SSH10	Putative selenium-binding protein (BAB40923)	*Oryza sativa* (*japonica* cultivar-group)	E= $2e^{-68}$	98
SSH11	Glutamine-dependent asparagine synthetase 1 (AAK49456).	*Hordeum vulgare* subsp. *vulgare*	E= $4e^{-39}$	90
SSH12	Hypothetical protein (XP_955549)	*Theileria annulata*	E= 4.4	36
SSH13	Light-harvesting chlorophyll a/b protein precursor (AAA33396)	*Lemna gibba*	E= $8e^{-44}$	97
SSH15	Hypothetical protein (BAC16191).	*Oryza sativa* (*japonica* cultivar-group)	E= 0.18	34
SSH16	Hypothetical protein (CAN65763)	*Vitis vinifera*	E= $3e^{-15}$	84

Table 4.2: Putative identities of the SSH fragments attained using the BLASTn algorithm (http://www.ncbi.nlm.nih.gov/blast).

Clone	*Closest homology (BLASTn)*	*Organism*	*Significance*	*Homology %*
SSH10	Putative selenium-binding protein (NM 001051729)	*Oryza sativa* (*japonica* cultivar-group)	E= $2e^{-138}$	91
SSH11	Glutamine-dependent asparagine synthetase 1 (AAK49456).	*Hordeum vulgare* subsp. *vulgare*	E= $1e^{-110}$	95
SSH12	No significant similarity found			
SSH13	cDNA clone (AK248764)	*Hordeum vulgare* subsp. *vulgare*	E= $2e^{-153}$	100
SSH15	Hypothetical protein (AK248318).	*Hordeum vulgare* subsp. *vulgare cDNA*	E= $2e^{-83}$	93
SSH16	28S ribosomal RNA gene (AY049041)	*Triticum aestivum*	E= $7e^{-66}$	99

BLASTn

```
SSH10          1 TTTGATCTTCAGCATGTCCAGGATGGTCTCTATGGCAGGCACCTGCATGTGTATGACTGG  60
                 |||||||||||||||| |||||||||||||||| || || ||||| ||||||||||||
NM001051729  773 TTTGATCTTCAGCATGTGCAGGATGGTCTCTATGGAAGACATCTGCACGTGTATGACTGG  832

SSH10         61 CCTGGTGGTGAGCTCAAGCAGACACTTGATCTAGGCAGCACAGGTCTTCTTCCGCTGGAG  120
                 |||||||||||||||||||||||||| ||| ||||||| |||||||||||||| || |||
NM001051729  833 CCTGGTGGTGAGCTCAAGCAGACACTAGATTTAGGCAGTACAGGTCTTCTTCCACTTGAG  892

SSH10        121 GTGAGGTTTTTACACGACCCATCAAAGGACACTGGCTATGTTGGCTGTGCTTTGACAAGC  180
                 |||||||||||||| || |||||||||| ||||| ||||| ||||||||| ||||||||
NM001051729  893 GTGAGGTTTTTACATGATCCATCAAAGGATACTGGGTATGTGGGCTGTGCTCTGACAAGC  952

SSH10        181 AACATGGTGAGGTTCTTCAAAACTGCAGATGGATCATGGAGCCATGAGGTGGCTATATCC  240
                 |||||||| || || ||||| ||||||||||||||||||||||||||||| ||||||||
NM001051729  953 AACATGGTCAGATTTTTCAAGACTGCAGATGGATCATGGAGCCATGAGGTTGCTATATCT  1012

SSH10        241   ATAGAACCATTGAAGGTGCGCAACTGGATGCTGCCAGAAATGCCAGGATTGATAACTGAT  300
                   ||| |||||||||| |||||||||||||| ||||| |||||||||||| ||||||||||
NM001051729 1013  ATAAAACCATTGAAAGTGCGCAACTGGATTCTGCCTGAAATGCCAGGACTGATAACTGAT 1072

SSH10        301   TTTGTCATCTCTCTCGATGACCGCTATCTCTACTTGGTTAATTGGCTTCATGGGGATATC  360
                   ||||| |||||||| ||||| |||||||||||||||| || |||||||||| ||||||
NM001051729 1073  TTTGTTATCTCTCTGGATGATCGCTATCTCTACTTGGTCAACTGGCTTCATGGTGATATC  1132

SSH10        361   AGGCAGTAC  369
                   |||||||||
NM001051729 1133  AGGCAGTAC  1141
```

BLASTx

```
SSH10          1  FDLQHVQDGLYGRHLHVYDWPGGELKQTLDLGSTGLLPLEVRFLHDPSKDTGYVGCALTS  180
                  FDLQHVQDGLYGRHLHVYDWPGGELKQTLDLGSTGLLPLEVRFLHDPSKDTGYVGCALTS
BAB40923     197  FDLQHVQDGLYGRHLHVYDWPGGELKQTLDLGSTGLLPLEVRFLHDPSKDTGYVGCALTS  256

SSH10        181  NMVRFFKTADGSWSHEVAISIEPLKVRNWMLPEMPGLITDFVISLDDRYLYLVNWLHGDI  360
                  NMVRFFKTADGSWSHEVAISI+PLKVRNW+LPEMPGLITDFVISLDDRYLYLVNWLHGDI
BAB40923     257  NMVRFFKTADGSWSHEVAISIKPLKVRNWILPEMPGLITDFVISLDDRYLYLVNWLHGDI  316

SSH10        361  RQY  369
                  RQY
BAB40923     317  RQY  319
```

Figure 4.5: Nucleotide and amino acid sequence alignment of SSH10 to its BLASTn and BLASTx best hits. (|) indicates similar nucleotides, ( ) indicates dissimilar nucleotides and amino acids (+) indicates different amino acids.

BLASTn

```
SSH11        1  GCTCGAGCTCTCGCGCAGGCTCAAGCACCGCGGCCCTGACTGGAGTGGCATGCACCAGGT  60
                ||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
AAK49456   134  GCTCGAGCTCTCGCGCAGGCTCAAGCACCGCGGCCCCGACTGGAGTGGCATGCACCAGGT  193

SSH11       61  CGGAGACTGCTACCTCTCCCACCGGCCCCTCGCCATCTTCCACCCTGCCTCGGGCGACCA  120
                ||| |||||||||||||||||| || |||||||||| || |||||||||| ||||||||
AAK49456   194  CGGTGACTGCTACCTCTCCCACCAGCGCCTCGCCATCATCGACCCTGCCTCAGGCGACCA  253

SSH11      121  GCCACTTTACAACGAGGACAAGTCCATCGTCGTCACTGTGAATGGAGAGATCTACATCCA  180
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
AAK49456   254  GCCACTTTACAACGAGGACAAGTCCATCGTCGTCACTGTGAATGGAGAGATCTACAACCA  313

SSH11      181  TGAACAGTTCCGGGCGCAGATCTCCTCCCACACTTTCAGGACAGGCAGCGACTGCGAGGT  240
                ||||||| |||||||||||| |||||||||||||| ||||||||||||||||||||||||
AAK49456   314  TGAACAGCTCCGGGCGCAGCTCTCCTCCCACACATTCAGGACAGGCAGCGACTGCGAGGT  373

SSH11      241  CATCGCAAACCTGTAC  256
                ||||||| ||||||||
AAK49456   374  CATCGCACACCTGTAC  389
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BLASTx

```
SSH11        2  LELSRRLKHRGPDWSGMHQVGDCYLSHRPLAIFHPASGDQPLYNEDKSIVVTVNGEIYIH  61
                LELSRRLKHRGPDWSGMHQVGDCYLSH+ LAI  PASGDQPLYNEDKSIVVTVNGEIY H
AAK49456    22  LELSRRLKHRGPDWSGMHQVGDCYLSHQRLAIIDPASGDQPLYNEDKSIVVTVNGEIYNH  81

SSH11       62  EQFRAQISSHTFRTGSDCEVIANLY  86
                EQ RAQ+SSHTFRTGSDCEVIA+LY
AAK49456    82  EQLRAQLSSHTFRTGSDCEVIAHLY  106
```


Figure 4.6: Nucleotide and amino acid sequence alignment of SSH11 to its BLASTn and BLASTx best hits. (|) indicates similar nucleotides, ( ) indicates dissimilar nucleotides and amino acids (+) indicates different amino acids.

BLASTx

```
SSH12      312 WENYPPTPWEK-GPNPGVNLKNTPLWGPMGEFNP----PRFPFG  196
               +  YPP P+    P+PG   + TP +GP G + P    P  P+G
XP_955549  192 YPGYPPVPYPPYQPHPGYQPQPTPQYGPYGPYQPYYPEPHQPYG  235
```

Figure 4.7: Amino acid sequence alignment of SSH12 to the BLASTx best hit. There was no homology to any nucleotide sequence found in GenBank. ( ) indicates dissimilar amino acids (+) indicates different amino acids.

BLASTn

```
SSH13        1 ACACACATACTCATGAGAACTCGATTTTCAAGTGTTTACTTGCCGGGCACGAAGTTGGTG  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AK248764   890 ACACACATACTCATGAGAACTCGATTTTCAAGTGTTTACTTGCCGGGCACGAAGTTGGTG  831

SSH13       61 GCGAAGGCCCACGCGTTGTTGTTGACGGGGTCGGCGAGGTGGTCAGCGAGGTTCTCGAGC  120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AK248764   830 GCGAAGGCCCACGCGTTGTTGTTGACGGGGTCGGCGAGGTGGTCAGCGAGGTTCTCGAGC  771

SSH13      121 GGGCCCTTACCAGTGACGATGGCTTGCACAAAGTATCCAAACATGGAAAACATTGCAAGA  180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AK248764   770 GGGCCCTTACCAGTGACGATGGCTTGCACAAAGTATCCAAACATGGAAAACATTGCAAGA  711

SSH13      181 CGACCGTTCTTGATCTCCTTCACCTTGAGCTCGGCAAACGCCTCGGGGTCGTCTGCAAGG  240
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AK248764   710 CGACCGTTCTTGATCTCCTTCACCTTGAGCTCGGCAAACGCCTCGGGGTCGTCTGCAAGG  651

SSH13      241 CCAAGGGGGTCGAAGCTGCCACCGGGGTATAGTGGGTCGACGATCTCTCCGAGTGGG  297
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AK248764   650 CCAAGGGGGTCGAAGCTGCCACCGGGGTATAGTGGGTCGACGATCTCTCCGAGTGGG  594
```

BLASTx

```
SSH13      296 PLGEIVDPLYPGGSFDPLGLADDPEAFAELKVKEIKNGRLAMFSMFGYFVQAIVTGKGPL  117
               PLGE+VDPLYPGGSFDPLGLADDPEAFAELKVKEIKNGRLAMFSMFG+FVQAIVTGKGPL
AAA33396   181 PLGEVVDPLYPGGSFDPLGLADDPEAFAELKVKEIKNGRLAMFSMFGFFVQAIVTGKGPL  240

SSH13      116 ENLADHLADPVNNNAWAFATNFVPGK  39
               ENLADHLADPVNNNAWAFATNFVPGK
AAA33396   241 ENLADHLADPVNNNAWAFATNFVPGK  266
```


Figure 4.8: Nucleotide and amino acid sequence alignment of SSH13 to its BLASTn and BLASTx best hits. (|) indicates similar nucleotides, ( ) indicates dissimilar nucleotides and amino acids (+) indicates different amino acids.

BLASTn

```
SSH15        2 GCCGGATGTAGTGACGTGGAGCGCCTCTAAGTCAGATATCCAAGCGTAGCGATGCGGACG  61
               ||||||| || |||| | || ||||||||||||||| |||||||| |||| |||| ||||
AK248318  2293 GCCGGAT-TA-TGAC-T-GAACGCCTCTAAGTCAGA-ATCCAAGC-TAGC-ATGC-GACG  2344

SSH15       62 CCGTGCGCCCGGCCGCTCGCCCGCGACCCACGTTAGGGGCGCTTGCGCCCCCAAGGGCCC  121
               || ||||||| |||||||||| ||||||||||||||||||||||||||||||||||||||
AK248318  2345 CC-TGCGCCC-GCCGCTCGCCC-CGACCCACGTTAGGGGCGCTTGCGCCCCCAAGGGCCC  2401

SSH15      122 GTGCCATGGGCTAAGTCGGTCCGGCCGATGTGCCGGTGATCGGCCGCCTCGAAGCTCCCT  181
               |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
AK248318  2402 GTGCCATGGGCTAAGTCGGTCCGGCCGATGTGCCG-TGATCGGCCGCCTCGAAGCTCCCT  2460

SSH15      182 TCCCAACGGGCGGTGGGCTGAATCCTTTGCAGACGA  217
               |||||||||||||||||||||||||||||||||||
AK248318  2461 TCCCAACGGGCGGTGGGCTGAATCCTTTGCAGACGA  2496
```

BLASTx

```
SSH15    295   GDGGIVSGRGGLAATIH*DPAPCRTDSSAKDSAHRPLGRELRGGRSPAHRPDRLSPWHGP  116
               G GG+ +GRG   A            S  +D   RP      GGRSPA  P   SP   P
BAC16191   8   GGGGMGAGRGLAVACRRRQDGGRARASGMEDGRRRPGAGRRDGGRSPAASP---SPPPDP  137

SSH15    115   LGAQAP--------LTWVAGERPGARRP  56
               G +A         L W  G   G RRP
BAC16191 138   AGGEAAGWRTTGGGLAWDGGMEAGCRRP  165
```

Figure 4.9: Nucleotide and amino acid sequence alignment of SSH15 to its BLASTn and BLASTx best hits. (|) indicates similar nucleotides, ( ) indicates dissimilar nucleotides and amino acids (+) indicates different amino acids.

```
BLASTn

SSH16        10 GGTACTTGTTCGCTATCGGTCTCTCGCCTGTATTTAGCCTTGGACGGAGTCTACCGCCCG  69
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AY049041    351 GGTACTTGTTCGCTATCGGTCTCTCGCCTGTATTTAGCCTTGGACGGAGTCTACCGCCCG  292

SSH16        70 ATTTGGGCTGCATTCCCAAACAACCCGACTCGTTGACCGCGCCTCGTGGGGCGACAGGGT  129
                |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
AY049041    291 ATTTGGGCTGCATTCCCAAACAACCCGACTCGTTGACGGCGCCTCGTGGGGCGACAGGGT  232

SSH16       130 CCGGGCCGGACGGGGCTCTCAC  151
                |||||||||||||||||||||
AY049041    231 CCGGGCCGGACGGGGCTCTCAC  210

BLASTx

SSH16       151 VRAPSGPDPVAPRGAVNESGCLGMQPKSGGRLRPRLNTGERPIANK  14
                VRAPS PDPVAPRGAV ESGC GMQ +SGG+ RPRLNTGERPIANK
CAN65763    285 VRAPSCPDPVAPRGAVGESGCXGMQXQSGGKFRPRLNTGERPIANK  330
```

Figure 4.10: Nucleotide and amino acid sequence alignment of SSH16 to its BLASTn and BLASTx best hits. (|) indicates similar nucleotides, ( ) indicates dissimilar nucleotides and amino acids (+) indicates different amino acids.

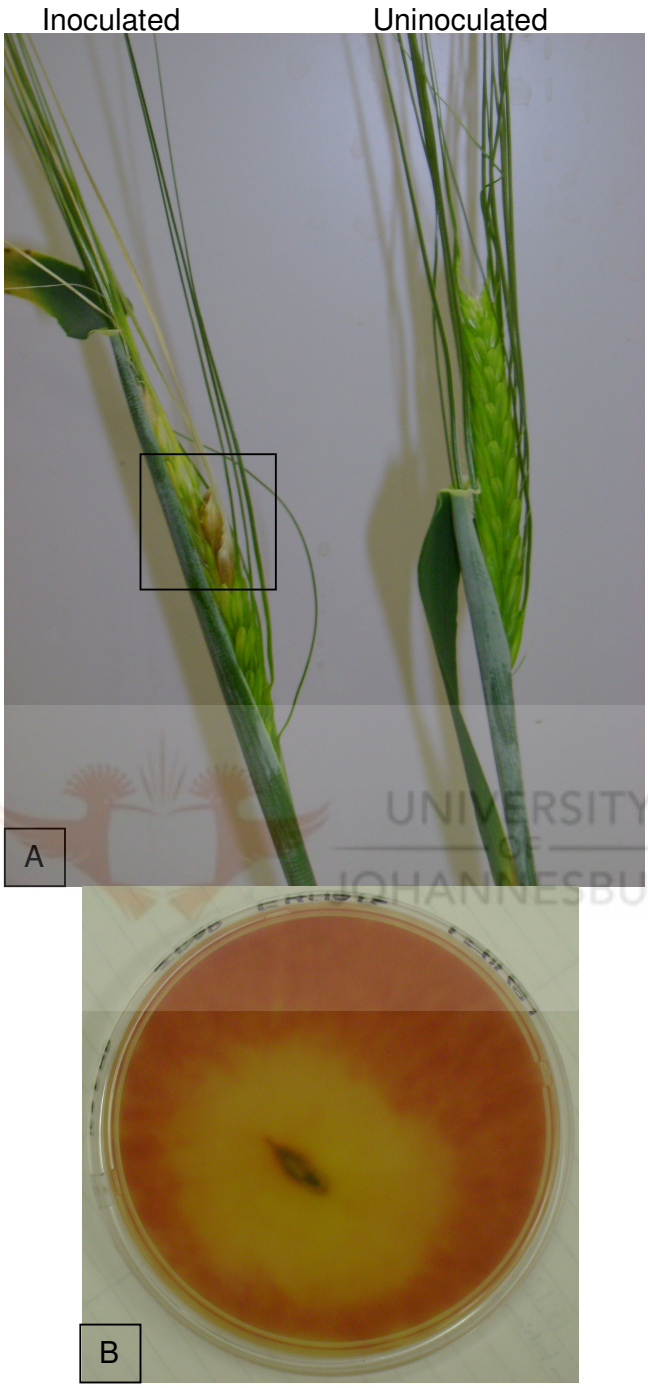

Figure 4.11: Pathogenicity results of barley infected with *F. graminearum.* (A) Visible decolourisation of the young kernels (indicated by block) after inoculation of a susceptible cultivar with *F. graminearum*. The control plant had no sign of infection on the heads. (B) Indicates the growth of *F. graminearum* on PDA medium after re-isolation from the plant material. The carmine red colony colour of *F.graminearum* is one of the characteristics of this fungus.

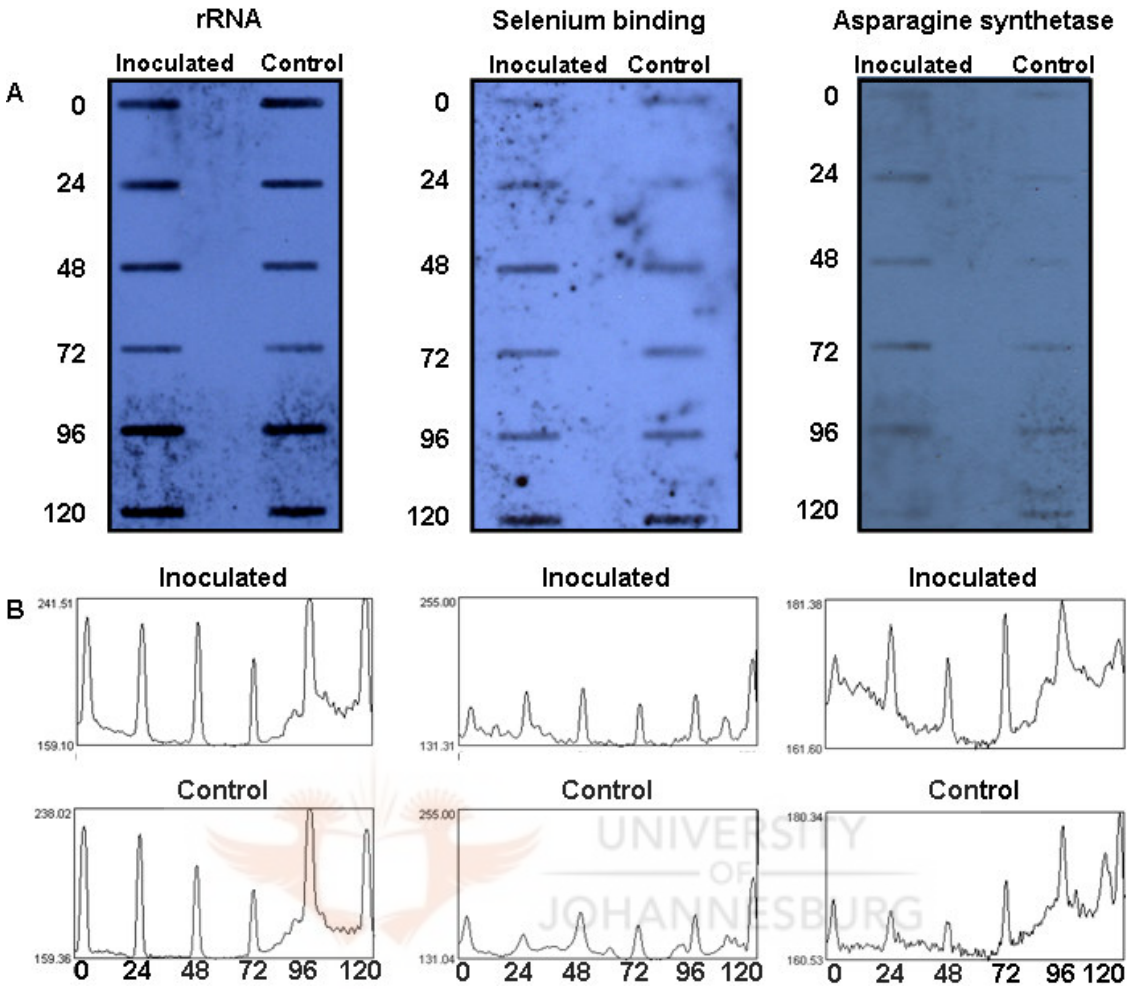

Figure 4.12: Expression analysis of selected TDFs. (A) Northern blot analyses to determine expression levels for each TDF over time. (B) Density profiles of each expression profile as plotted by Scion Image (Scion Inc.). These profiles indicate the density profile plot for each of the individual bands on the Northern blots, confirming the visual observation for each of the Northern blots. Scales are allocated by the program and could not be changed.