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Gene expression analysis of the wheat response to infection by *Fusarium pseudograminearum*

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ABSTRACT

Crown rot (CR) of wheat, caused by *Fusarium pseudograminearum* (*Fp*) and other *Fusarium* species, is an important disease globally. To understand the host response to challenge by *Fp*, we examined gene expression changes in the wheat stem base following inoculation with macroconidia using the Affymetrix GeneChip Wheat Genome Array. Induced genes included mainly those with defensive functions such as genes encoding anti-microbial proteins as well as oxidative stress-related proteins, signalling molecules, and proteins involved in both primary and secondary metabolism. Comparison of genes induced by *Fp* and the biotrophic rust pathogen *Puccinia triticina* revealed substantial overlap in most functional classes of induced genes, except for oxidative stress-related genes which were specifically induced by the necrotroph, *Fp*. Differential expression of selected *Fp*-induced genes was confirmed and further analysed using real-time quantitative RT-PCR on an inoculation time-course of wheat cultivars Kennedy and Sunco. Interestingly, several genes were induced earlier, and to higher levels, in the partially CR-resistant cultivar Sunco than in susceptible Kennedy. Many *Fp*-induced genes were also activated by methyl jasmonate and benzothiadiazole, an analogue of salicylic acid, suggesting that these signalling molecules may be involved in activating defences during crown rot. Most of the genes identified here that were induced by *Fp* were also induced by deoxynivalenol (DON), a toxin produced by *Fp* during CR. In particular, DON induced several genes encoding glucosyltransferases that may be involved in DON detoxification. To initiate functional characterisation, one of these wheat glucosyltransferase genes was over-expressed in *Arabidopsis thaliana*, however this did not result in improved tolerance to DON. This study is the first comprehensive analysis of the wheat transcriptome during CR and provides new insights into the host processes potentially involved in plant defence against this pathogen.

1. Introduction

Crown rot (CR) caused by *Fusarium* pathogens is emerging as a serious disease of wheat (*Triticum aestivum*) globally (Ref. [1] and refs cited therein). Visible symptoms include the formation of brown lesions at the crown and stem base. This necrosis can impede transport of water and nutrients up the stem and in severe cases results in the formation of 'whiteheads' which are heads that are empty or contain shrivelled and sub-standard grain [2]. In Australia, CR is predominately caused by the fungal pathogen *Fusarium pseudograminearum* (*Fp*). This pathogen is thought to be a necrotroph during infection of wheat, but can also grow saprophytically on debris. Therefore, environmentally conservative farming practices such as minimum tillage have recently resulted in increased incidence of CR [3]. Currently, the main methods for controlling this disease are crop rotations and the use of resistant wheat cultivars such as Sunco [4]. However, current resistance in commercial varieties is only partial and disease outbreaks are common and can be severe when climatic conditions are favourable for the pathogen [5]. Improving resistance would be a great advantage for farming areas where this disease causes significant financial losses.

Fp is closely related to *F. graminearum* (*Fg*) and both of these pathogens are able to cause *Fusarium* head blight (FHB) and CR diseases [6,7], and produce the mycotoxin deoxynivalenol (DON) during infection [7,8]. The role of DON during FHB has been extensively characterised and shown to be necessary for spread of the fungus from infected florets into the rachis prior to further colonisation of the wheat head [9–11]. In addition, wheat cultivars that accumulate less DON, possibly due to their ability to detoxify this toxin, have been shown to be more resistant to FHB infection

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[12,13]. Comparatively little is known about the role of DON during CR, but it seems to be needed for fungal colonisation of upper stem nodes following infection at the crown and stem base [8].

Inherent differences in disease resistance that exists in different cultivars of the same species can sometimes be traced back to variations in the expression of host genes, particularly those with defensive functions [14]. Genetic manipulation of genes involved in pathogen defence responses has frequently been demonstrated to improve disease resistance in a range of plants [15–20]. In wheat, over-expression of the well-known defence gene *PR2*, encoding β-1,3-glucanase, has resulted in reduced FHB severity [21]. Transgenic approaches that pyramid a range of defence genes have also achieved improved disease resistance, sometimes more effectively and with more stable and longer lasting effects than the individual genes concerned [22–26]. To maximise the chance of improving disease resistance using transgenic approaches, a good understanding of the host–pathogen interaction is needed, as this will allow a logical selection of candidate genes for manipulation.

In contrast to the large-scale analyses of plant defence gene expression in wheat and barley heads after infection with *Fg* [27–30], very little work has been done on plant responses to the closely related species *Fp* after the infection of wheat crown tissue. Large-scale analysis of gene expression can provide information on potential host functions that may be activated in response to pathogen challenge, provide clues to the signalling processes involved and suggest the type of physiological stresses that pathogen invasion may inflict on the host [31]. Therefore, one aim of the current investigation is to characterise host gene expression changes following infection of wheat stem tissue with *Fp* at the early stages of CR disease onset. From small-scale studies using real-time RT-qPCR analysis of selected genes, it is known that a range of defence-related genes such as *PR1*, *PR2*, *PR3*, *PR4*, *PR5*, and *peroxidase* are induced in stem tissue within 4 days after inoculation, and furthermore, induction of these genes occurs earlier in the partially CR-resistant variety Sunco than the susceptible variety Kennedy [32].

The recent release of the GeneChip® Wheat Genome Array (http://www.affymetrix.com/products/arrays/specific/wheat.affx) has provided a powerful resource for characterising the host response at the gene expression level in CR disease. This tool contains 61,127 probe sets representing 55,052 wheat gene transcripts and we have used it to assess gene expression changes that occur after *Fp* inoculation of wheat stem tissue. Our results show induction of many genes that have previously been linked with host–pathogen interactions, for example, genes encoding antimicrobial proteins. In addition, further wheat genes that have not commonly been associated with pathogen defence responses were identified and these may represent uncharacterised components of the defence response activated in wheat during CR. The expression of several selected *Fp*-inducible genes was also found to be responsive to the *Fusarium* mycotoxin DON and the signalling compounds methyl jasmonate (MJ) and benzothiadiazole-7-carbothionic acid S-methyl ester (BTH), an analogue of salicylic acid. The genes identified here are likely to encode components of the defence response activated in wheat during CR and their manipulation may provide potential avenues for improving CR resistance.

2. Materials and methods

2.1. Plant material and fungal inoculation

The *Fusarium* susceptible wheat cultivar Kennedy was used for gene expression analysis using the Affymetrix GeneChip Wheat Genome Array. Plants were grown in glasshouse conditions as previously described [32]. *Fp* isolate CS3096 was used for all inoculations. This fungus was grown on ½ PDA media. Spores were collected and 10 μL of inoculum containing 10^6 spores per mL was applied to the stem base of 2-week-old wheat seedlings as previously described [32].

2.2. Sampling and RNA extraction for Affymetrix chip hybridisation

Inoculated and mock-inoculated stem tissues were sampled one day after treatment by collecting three independent biological replicates, each consisting of the lower 2 cm, from the soil level up, of ~20 stems. Samples were frozen immediately after sampling and in this state they were ground to a fine powder using a mortar and pestle. Total RNA was isolated using a caesium chloride extraction method [33] followed by a DNase treatment using an RNase-free DNase kit (Qiagen, Hilden, Germany) and column purification using a MinElute kit (Qiagen) both according to manufacturer's instructions. Samples were dissolved in nuclease free water and sent to the Australian Genome Research Facility (Parkville, Victoria, Australia) for cDNA synthesis, labelling, hybridisation to the wheat Affymetrix GeneChip Wheat Genome Array (Affymetrix, Santa Clara, CA, USA), washing and scanning. Details of the wheat Affymetrix GeneChip Wheat Genome Array can be found at http://www.affymetrix.com/products/arrays/specific/wheat.affx.

2.3. Affymetrix chip data analysis

Resulting Affymetrix data files are publically available from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under the accession number of GSE13660. Data analysis was done using GeneSpring 7.3 (Agilent Technologies) using methods adapted from Casu et al. [34]. Raw data was normalised by dividing each probe set value by the median of that probe set from all samples, effectively centering the data around 1 and enabling simple identification of differentially expressed genes. Statistical analysis was performed to identify genes that were differentially expressed in *Fp*-inoculated samples compared to mock-inoculated samples using an Analysis of Variance (ANOVA, $p < 0.05$) across all replicates. Induction or repression of significantly differentially expressed genes was determined by dividing the raw signal value for each replicate from *Fp*-inoculated tissue by the average of the raw signal values from mock-inoculated controls. Genes were then classed as 'induced' or 'repressed' if their change in expression was >1.5-fold. Bioinformatic analysis of induced and repressed genes was done using a batch BLAST and putative functions assigned if the *E*-value was less than $1e^{-10}$. Alignments with a higher score were visually inspected and annotated if a reasonable degree of homology was observed. Genes were classed as unknown if no reasonable alignments were found. Duplicate probe sets for any particular UniGene ID were removed from final presented data except for the probe set with the highest raw signal in control samples.

Publicly available datasets for wheat (cv. Thatcher) inoculated with the fungal biotroph *Puccinia triticina* were obtained from http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE6227 [35]. Six datasets were used including GSM143502–GSM143504 from Thatcher basal leaf inoculated samples and GSM143508–GSM143510 from Thatcher basal leaf mock-inoculated controls. Datasets were analysed in the same way as *Fp*-inoculated and mock-inoculated Kennedy samples.

2.4. Gene expression analysis using real-time quantitative PCR (RT-qPCR)

RT-qPCR confirmation of expression for selected genes was done using both Kennedy and Sunco cultivars. Two-week-old

seedlings were treated with 300 μM MJ and BTH (in the form of BION™) and sampled in three biological replicates as previously described [32]. Samples were treated with DON using the same procedure as that described for inoculation except using a solution of 100 mg L^{-1} DON instead of inoculum. Samples of 8–10 stems were collected in three biological replicates. All other procedures for RNA extraction, cDNA synthesis, and RT-qPCR were the same as previously described [32]. RT-qPCR primer sequences are listed in Supplementary Table 1.

2.5. Construction of the binary vector and Arabidopsis transformation

The full coding sequence of the glucosyltransferase gene (BT009372) was amplified from Sunco, Kennedy, Sumai 3, and Chinese Spring using the primers GT3BS(HindIII)-F (5′-CAGAAGCTTCAACCATGACCTTC-3′) and GT3BS(HindIII)-R (5′-ACAAGCTTTGTTTACACCAAAAAAGAG-3′). The sequence of this gene was identical for each wheat cultivar and the amplification product from Sumai 3, a well-known *Fusarium* resistant wheat cultivar, was used. The PCR amplification product was initially cloned into pBLUNT (Invitrogen, Carlsbad, CA) according to manufacturer's instructions and then removed using HindIII restriction digest followed by blunt-ended ligation into the binary vector pKEN using the procedure previously described [36]. *Arabidopsis* floral dip was then used to produce transgenic lines over-expressing the wheat glucosyltransferase as well as the selectable marker gene, *bar*, encoding basta resistance. Homozygous T2 generation lines were identified by screening their progeny, the T3 generation, for segregation of basta resistance. Ten independent T1 lines were found to be homozygous, and the level of glucosyltransferase over-expression in each line was quantified using RT-qPCR with primers listed in Supplementary Table 1 (BT009372). Transgenic *Arabidopsis* lines were tested for tolerance to DON by surface sterilizing seed which was then plated out on ½ MS media containing 5 mg L^{-1} DON and kept at 4 °C for 3 days. The plates were incubated in a controlled environment growth room under 300 μmol m^{-2} s^{-1} photosynthetically active radiation during an 8 h-photoperiod. Air temperature was maintained at 24/19 °C day/night with 65/95% relative humidity, respectively. Col-0 WT seedlings were grown on the same plate as each over-expression line for comparison. Plant growth, in particular root development, was monitored over the following month.

3. Results

3.1. Fp elicited gene expression changes in wheat assessed using Affymetrix chips

Large-scale gene expression analysis is increasingly used to characterise how plants respond to their environment. *Fp* spores were inoculated onto wheat tissue and germinated within 6 h. Within one day, extensive hyphal growth was observed on the surface of inoculated tissue (data not shown). Using the Affymetrix GeneChip platform, we determined gene induction and repression in the stem base one day after inoculation with conidia of *Fp*. In total, there were 1248 unique genes induced and 1497 unique genes repressed (ANOVA, $p < 0.05$) when compared to mock-treated controls. Amongst these, 213 genes were induced more than 1.5-fold and only nine genes were repressed more than 1.5-fold. A bioinformatic analysis was undertaken to assign a description and functional classification to these genes and results are shown in Supplementary Tables 2 and 3, respectively. The nine genes that were at least 1.5-fold repressed encode a photosystem II type I chlorophyll *a*/*b* binding protein and a peptidylprolyl isomerase and the remaining seven genes were of unknown function. Putative functions could be assigned to most of the induced genes and these have been represented using a pie chart to show the functional distribution of the host transcriptional response (Fig. 1).

The largest class of induced genes contained those encoding anti-microbial proteins directly implicated in plant defence during pathogen attack, such as chitinase, beta-1,3-glucanase, PR1, PR10, and thaumatin-like proteins. In addition, several genes were identified that encode proteins such as phenylalanine ammonia-lyase and chalcone synthase involved in the synthesis of small anti-microbial molecules such as phytoalexins [37,38]. Activation of anti-microbial defences requires signalling molecules and transcription factors and our analysis identified several *Fp*-inducible genes that appear to encode regulatory proteins, such as protein kinases, and transcription factors containing basic helix-loop-helix or WRKY DNA-binding domains identified here (Supplementary Tables 2 and 4, Fig. 2).

Genes encoding peroxidases and germin-like proteins were among those induced and are involved in the metabolism of reactive oxygen species (ROS) [39]. In turn ROS molecules such as superoxide and hydrogen peroxide are known to be involved in signalling for programmed cell death during pathogen defence responses [40,41]. In keeping with this concept, we observed strong

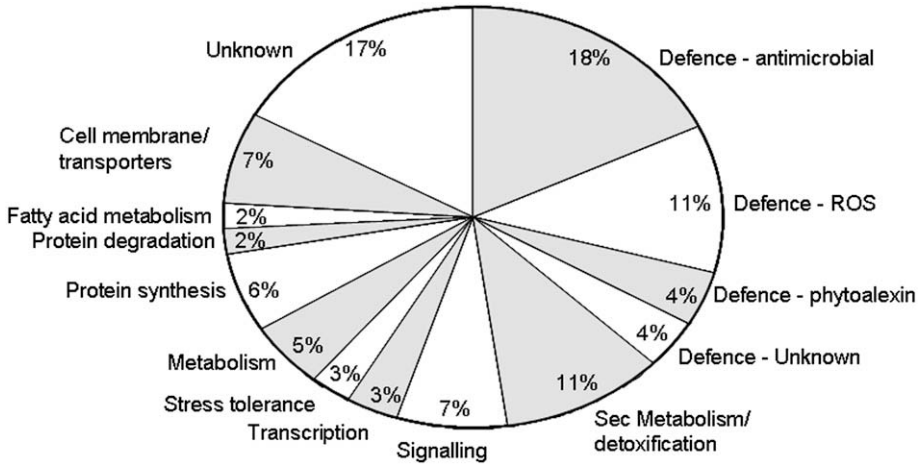

Fig. 1. Functional classes of genes significantly induced at a level of >1.5-fold compared to mock-treated controls 1 day after *Fusarium pseudograminearum* inoculation.

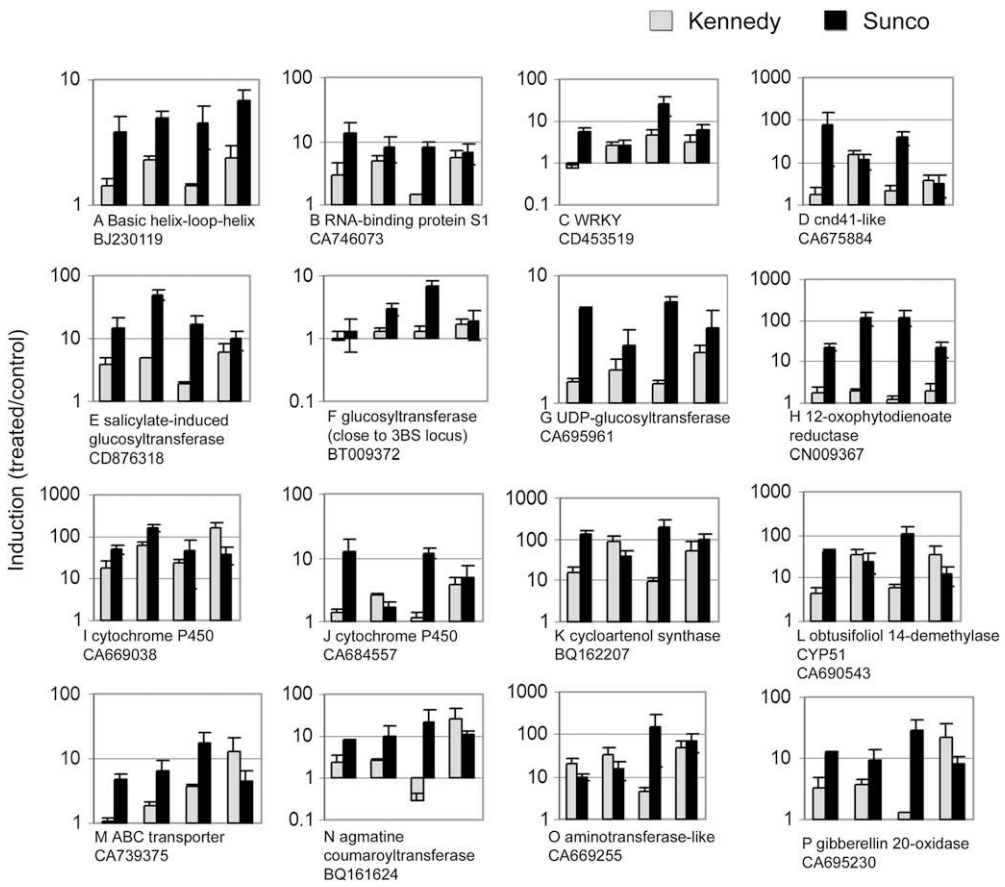

Fig. 2. Gene induction (fold change in inoculated compared to control samples) in Sunco and Kennedy 6 h, 1 day, 2 days, and 4 days (left to right for each profile) post-inoculation with *Fusarium pseudograminearum*. Gene transcript levels were determined using RT-qPCR. Columns represent average induction (+SE, $n = 3$) plotted on a logarithmic scale. The description and Genbank accession number for each gene is given below its profile. Numerical induction values for all genes assessed are shown in the Supplementary Table 4.

induction of a gene encoding a *cnd41*-like gene in wheat (Supplementary Tables 2 and 4, Fig. 2). Previously cnd41 was found to be functioning in rubisco degradation and the translocation of nitrogen during senescence [42].

Another large functional group of induced genes represented those involved in secondary metabolism. This group included genes encoding cytochrome *P*450 proteins as well several glucosyltransferases that are known to be involved in detoxification of exogenous compounds [43], and may be involved in protecting host cells from fungal toxins such as DON produced by *Fp* [8,44].

3.2. Gene expression analysis using RT-qPCR following inoculation

A group of genes that included members from all of the functional classes of induced genes from the global gene expression data obtained using the Affymetrix wheat GeneChip was selected for further analysis. Expression profiling of these genes was extended using RT-qPCR on samples of both Sunco and Kennedy collected at 6 h, 1 day, 2 days and 4 days following *Fp* inoculation. Differences in gene expression changes between these two cultivars may indicate particular genes that contribute to the higher level of CR resistance observed in Sunco [4].

Several defence genes showed greater and/or earlier induction in the partially crown rot resistant cultivar Sunco compared to the susceptible cultivar Kennedy, while genes that were induced more in Kennedy over the whole time-course were rare (Fig. 2, Supplementary Table 4). This is consistent with previous work showing induction of several PR defence genes occurred earlier in Sunco compared to Kennedy [32]. In particular, the present work identified genes involved in regulating transcription, and secondary metabolism genes such as glucosyltransferases and 12-oxophytodienoate reductase (12-OPDR) that were substantially more induced in Sunco compared to Kennedy (Fig. 2).

Interestingly, one of the genes induced particularly strongly in Sunco encodes an agmatine coumaroyltransferase (ACT) (Fig. 2). This enzyme catalyses the first step in the biosynthesis of antifungal hydroxycinnamoylagmatine derivatives, and in barley ACT has been shown to produce the precursor of hordatine, a proven antifungal compound [45]. Although hordatine production seems to be restricted to barley, the presence of antifungal hydroxycinnamoylagmatine derivatives in wheat has been previously reported [46]. Similarly, genes encoding cycloartenol synthase (BQ162207) and obtusifoliol 4 demethylase, a member of the CYP51 cytochrome *P*450 gene family (CA690543), were induced to higher levels by *Fp* in Sunco (Fig. 2). The enzymes encoded by possible oat homologs of both of these genes are involved in plant sterol biosynthesis and in the synthesis of the anti-microbial compound avenacin in oat roots [47]. Further research is required to determine the actual defensive function of these genes in wheat.

Gene expression analysis of selected genes was also done following treatment with the signalling compounds MJ and BTH, an analogue of salicylic acid, Table 1. Both of these treatments activated several *Fp*-inducible genes suggesting they may be involved in defence responses induced during CR. In contrast to defence gene induction by the pathogen, there was not a general trend of higher induction in Sunco compared to Kennedy following these chemical treatments.

Table 1
Average gene induction of selected genes 1 day after *Fusarium pseudograminearum* inoculation, treatment with DON (deoxynivalenol), MJ (methyl jasmonate), or BTH (benzothiadiazole; SA analogue). Values represent average fold change of gene transcripts, with SE in parenthesis, in inoculated compared to control samples for 3 biological replicates. Genes highlighted in bold were found to be induced after Fp inoculation, but not after *Puccinia triticina* inoculation.

Genbank	Description	Affymetrix data	RT-qPCR data					
		Fp	DON 100 mg L^{-1} 1 day		MJ 300 μM 1 day		BTH 1 day	
		Kennedy	Kennedy	Sunco	Kennedy	Sunco	Kennedy	Sunco
		Average (SE)	Average (SE)	Average (SE)	Average (SE)	Average (SE)	Average (SE)	Average (SE)
Defence – anti-microbial								
CD863039	Thaumatin-like protein	20.0 (4.10)	9.9 (0.38)	4.4 (0.47)	18.7 (0.47)	1.2 (0.48)	1.38 (0.37)	0.08 (0.02)
CK205943	Chitinase	12.4 (3.43)	6.5 (0.77)	5.0 (0.66)	8.9 (0.19)	3.3 (0.79)	3.21 (0.69)	0.19 (0.01)
Defence – ROS								
Y09916	**Germin-like protein**	**5.1 (0.96)**	**10.3 (1.65)**	**13.6 (2.70)**	**0.7 (0.07)**	**1.8 (1.40)**	**2.82 (0.82)**	**0.39 (0.03)**
AF031195	Blue copper-binding protein	2.2 (0.29)	10.0 (1.86)	25.7 (1.76)	1.9 (0.07)	3.4 (0.83)	1.88 (0.43)	0.41 (0.06)
CA667447.1	Germin-like protein	1.9 (0.15)	7.0 (1.79)	5.3 (0.86)	0.8 (0.02)	1.5 (0.56)	2.66 (0.59)	0.55 (0.12)
BQ165963	Peroxidase	3.3 (0.88)	17.9 (2.26)	33.5 (2.67)	1.2 (0.02)	1.2 (0.42)	2.15 (0.55)	0.09 (0.02)
Defence – phytoalexin								
BQ161624	Agmatine coumaroyltransferase	3.0 (0.56)	7.3 (0.90)	29.8 (11.88)	26.6 (6.95)	7.8 (1.59)	0.56 (0.13)	0.02 (0.00)
CA682712	Flavonoid 7-O-methyltransferase-like	2.6 (0.52)	25.9 (11.73)	35.7 (6.41)	21.8 (2.10)	2.6 (1.31)	0.28 (0.05)	0.05 (0.03)
Defence – unknown								
CA684533	WIR1B	24.2 (5.74)	2.0 (0.15)	1.0 (0.20)	1.5 (0.16)	1.2 (0.46)	1.06 (0.20)	0.48 (0.12)
U32431	**WCI-5**	**3.1 (0.36)**	**6.5 (0.49)**	**4.2 (0.28)**	**0.5 (0.10)**	**0.7 (0.25)**	**0.22 (0.02)**	**0.45 (0.04)**
Sec metabolism/detoxification								
CA690543	Obtusifoliol-14-demethylase (CYP51) like	21.2 (4.92)	38.2 (3.66)	32.9 (5.73)	9.1 (0.63)	4.5 (1.63)	0.81 (0.29)	0.10 (0.02)
BQ162207	Cycloartenol synthase	16.0 (3.78)	46.7 (2.83)	11.0 (7.55)	16.1 (3.24)	2.6 (1.36)	0.47 (0.32)	0.05 (0.03)
CD876318	Salicylate-induced glucosyltransferase	14.7 (4.11)	3.0 (0.29)	4.3 (1.19)	3.9 (0.42)	0.7 (0.36)	0.48 (0.03)	0.03 (0.01)
CA669038	Cytochrome *P*450	14.6 (5.38)	10.3 (0.85)	18.1 (2.13)	22.7 (0.23)	1.2 (0.57)	1.77 (0.42)	0.02 (0.01)
CA683802	Obtusifoliol-14-demethylase	7.0 (2.48)	60.5 (3.23)	27.4 (5.21)	77.2 (9.84)	3.0 (1.97)	2.92 (0.05)	0.08 (0.04)
CA684557.1	Cytochrome *P*450	3.4 (0.83)	5.5 (0.89)	3.2 (0.55)	0.3 (0.03)	1.9 (0.37)	1.33 (0.31)	0.34 (0.10)
CK199685	**Glucosyltransferase**	**2.8 (0.62)**	**7.8 (0.72)**	**14.8 (1.83)**	**0.7 (0.08)**	**0.9 (0.42)**	**0.53 (0.22)**	**0.09 (0.03)**
CN009367	**12-Oxophytodienoate reductase**	**2.7 (0.25)**	**10.7 (1.35)**	**13.3 (1.88)**	**8.2 (0.15)**	**3.4 (1.81)**	**3.37 (0.51)**	**1.52 (0.34)**
CA695961	UDP-glucosyltransferase family protein	2.6 (0.57)	5.8 (1.11)	7.5 (0.59)	0.7 (0.28)	2.9 (0.88)	1.65 (0.39)	0.89 (0.22)
BT009372.1	Glucosyltransferase near 3BS locus	2.0 (0.26)	5.6 (0.93)	8.6 (1.00)	1.5 (0.12)	**2.3** (0.87)	1.73 (0.35)	**1.08** (0.23)
BJ250503	Putative glucosyltransferase-10	3.2 (0.86)	3.0 (0.42)	15.7 (1.48)	1.6 (0.02)	1.6 (0.50)	1.74 (0.56)	0.10 (0.03)
Signalling								
BQ237026	**Putative serine/threonine kinase protein**	**1.7 (0.17)**	**2.5 (0.21)**	**1.7 (0.75)**	**1.5 (0.29)**	**1.2 (0.34)**	**1.38 (0.40)**	**0.93 (0.21)**
CA637923	**Receptor protein kinase-like protein**	**1.5 (0.04)**	**1.3 (0.73)**	**0.7 (0.23)**	**19.3 (17.28)**	**1.3 (0.31)**	**6.88 (1.60)**	**1.17 (0.13)**
CA695230	Leucine rich repeat containing protein kinase	5.1 (1.49)	21.0 (2.10)	20.3 (2.36)	2.5 (0.19)	5.5 (0.70)	0.17 (0.08)	0.25 (0.14)
Transcription								
BJ230119	**Basic helix-loop-helix**	**2.5 (0.39)**	**11.3 (1.23)**	**12.5 (0.76)**	**0.8 (0.15)**	**0.7 (0.07)**	**0.96 (0.14)**	**0.59 (0.11)**
CA644335	**EBNA-1 nuclear protein**	**2.1 (0.38)**	**9.7 (1.10)**	**14.3 (0.35)**	**6.0 (0.10)**	**1.6 (0.55)**	**6.69 (0.32)**	**0.38 (0.10)**
CA746073	RNA-binding protein S1	1.8 (0.11)	5.7 (0.45)	3.7 (0.38)	1.1 (0.30)	0.7 (0.39)	0.96 (0.18)	0.19 (0.05)
CA675884	Chloroplast nucleoid DNA-binding protein cnd41-like	2.5 (0.35)	0.7 (0.09)	0.5 (0.10)	0.4 (0.10)	0.6 (0.32)	0.49 (0.08)	0.40 (0.07)
BQ168535	PORF1	1.5 (0.06)	3.4 (0.02)	6.6 (1.14)	59.4 (7.67)	5.5 (2.25)	2.67 (0.43)	0.11 (0.03)
CD453519	Unknown protein, contains WRKY DNA-binding domain	1.3 (0.05)	1.3 (0.32)	4.3 (0.17)	11.7 (0.08)	1.2 (0.22)	1.56 (0.47)	0.14 (0.02)
CA637851	Basic helix-loop-helix (BHLH) family protein-like	1.5 (0.14)	2.8 (0.48)	6.1 (0.68)	0.6 (0.30)	1.2 (0.20)	1.23 (0.28)	1.08 (0.17)
Stress tolerance								
CA694095	wali3	3.4 (0.70)	1.1 (0.06)	0.9 (0.18)	4.2 (0.22)	4.3 (3.31)	0.21 (0.00)	0.36 (0.06)
BG907881	**Heat shock factor protein hsf8-like**	**2.3 (0.07)**	**1.0 (0.28)**	**0.6 (0.58)**	**0.4 (0.17)**	**0.2 (0.03)**	**0.70 (0.10)**	**0.38 (0.15)**
L11882	**wali5**	**1.8 (0.18)**	**4.1 (0.29)**	**3.4 (0.36)**	**0.9 (0.12)**	**2.5 (1.37)**	**0.35 (0.01)**	**0.14 (0.04)**
Metabolism								
CA669255	**Aminotransferase**	**7.4 (0.61)**	**62.2 (3.93)**	**45.0 (15.66)**	**9.1 (0.60)**	**1.2 (0.69)**	**0.12 (0.01)**	**0.05 (0.04)**
Protein synthesis								
CA690208	**60S Acidic ribosomal protein P0-A**	**2.7 (0.54)**	**1.8 (0.02)**	**1.7 (0.52)**	**0.6 (0.01)**	**2.1 (0.37)**	**1.29 (0.16)**	**0.32 (0.12)**
Protein degradation								
CA667787	Subtilisin-like serine proteinase	2.3 (0.37)	4.4 (0.20)	3.3 (0.47)	3.4 (0.02)	2.4 (1.04)	1.56 (0.00)	0.36 (0.09)
CK195059	**Cysteine proteinase**	**2.3 (0.40)**	**9.1 (1.10)**	**1.6 (0.84)**	**0.7 (0.33)**	**0.6 (0.24)**	**0.68 (0.34)**	**0.09 (0.05)**
Fatty acid metabolism and derivatives								
BE412340	**Patatin-like protein**	**3.6 (0.92)**	**6.9 (0.87)**	**7.8 (1.10)**	**3.0 (0.34)**	**2.8 (1.45)**	**4.82 (1.12)**	**0.28 (0.04)**
Cell membrane/transporters								
BQ162027	PDR-like ABC transporter	4.5 (1.36)	27.5 (1.27)	13.8 (2.88)	8.4 (1.21)	0.5 (0.14)	0.69 (0.25)	0.09 (0.05)
CA739375	ABC transporter-like	2.9 (0.56)	8.4 (1.23)	4.3 (0.67)	0.3 (0.07)	1.2 (0.23)	0.83 (0.15)	0.38 (0.06)
CD883484	Monosaccharide transporter 4	2.5 (0.29)	17.7 (2.00)	28.1 (4.87)	1.9 (0.19)	1.4 (0.45)	1.78 (0.29)	0.99 (0.19)
CA733231	**Glutathione transferase**	**1.9 (0.18)**	**2.8 (0.64)**	**4.3 (0.50)**	**68.5 (2.37)**	**4.3 (1.74)**	**36.24 (11.36)**	**0.45 (0.18)**
BJ285699	**Syntaxin**	**1.6 (0.18)**	**2.6 (0.16)**	**3.1 (0.25)**	**1.3 (0.19)**	**1.8 (0.20)**	**1.50 (0.13)**	**0.90 (0.14)**
Unknown								
CA686527	Transcribed locus	3.2 (0.64)	2.0 (1.61)	0.5 (0.19)	5.6 (0.15)	1.2 (1.14)	1.24 (0.25)	0.08 (0.01)
AF079526	Secretory protein (WAS-2)	1.5 (0.11)	1.4 (0.18)	1.7 (0.21)	1.7 (0.05)	1.2 (0.45)	1.28 (0.18)	0.13 (0.04)

Most of the genes induced by *Fp* were also induced by DON consistent with previous results that suggest DON is able to activate defence responses in wheat plants [40]. Glucosyltransferases are thought to be responsible for detoxification of exogenous compounds. For DON, this involves the formation of the conjugation product DON-3-O-glucoside, which is less toxic than DON itself [48]. Interestingly, several glucosyltransferase genes were more highly induced in Sunco than Kennedy (Table 1). The glucosyltransferase genes identified here that were induced by both *Fp* and treatment with 100 mg L^{-1} DON, particularly those that were induced more in Sunco compared to Kennedy (Supplementary Table 4), may be good candidates for manipulation to improve tolerance to DON.

Transformation of wheat is a lengthy process that involves production of embryogenic callus followed by transformation using microprojectile bombardment or *Agrobacterium*-mediated transformation techniques [49,50]. It is further complicated by the limited number of varieties that can be used to produce transgenic lines that show stable expression of the transgene [51]. Due to these difficulties in transformation of wheat, we have used an alternative strategy to functionally characterise a wheat glucosyltransferase gene (BT009372) by over-expression in the easily transformed model species *Arabidopsis thaliana*. Previous work on *Arabidopsis* lines over-expressing an endogenous glucosyltransferase gene found these lines were more tolerant to growth on media containing DON [44]. The wheat glucosyltransferase gene we selected was induced by both the pathogen and the DON, in both wheat cultivars tested, with higher induction observed in Sunco (Supplementary Table 4). This gene was also found to be located near the 3BS FHB resistance locus [52] and in wheat, reduced accumulation of DON has been shown to co-localise with the major FHB resistance QTL 3BS [48]. *Arabidopsis* over-expression lines were tested for improved DON tolerance by assessing growth on media containing 5 mg L^{-1} DON. Although strong transgene expression was achieved in the 10 independent glucosyltransferase over-expression lines tested, we observed no difference in DON sensitivity compared to WT Col-0 plants when grown on media containing DON. Both WT and over-expression lines were stunted and had severely impaired root growth when grown in the presence of DON, compared to plants grown on media without DON (data not shown). These results suggest that the wheat glucosyltransferase tested here may not be functional in *Arabidopsis* or that DON may not be its preferred substrate. Functional analysis in wheat is required to confirm the role of this particular glucosyltransferase during crown rot disease.

3.3. Comparison of gene expression changes induced during CR and wheat leaf rust disease

Plants induce specific defence responses depending on the type of pathogen to which they are exposed [31,53]. Widespread use of a uniform gene expression analysis platform such as the Affymetrix GeneChip means that more robust comparisons of gene expression analysis are now possible based on publicly available results from groups working on these different types of pathogens. The first and only other work describing host–pathogen interactions using the Affymetrix GeneChip Wheat Genome Array have assessed gene expression changes in wheat lines that are near-isogenic except for the *Lr34*/*Yr18* rust resistance gene following inoculation with the biotrophic pathogen *P. triticina*, causal agent of wheat leaf rust [35]. Comparing the genes induced by a biotrophic fungal pathogen such as *P. triticina* to those induced by a necrotrophic pathogen such as *Fp*, may identify genes unique to a particular pathogen type and infection strategy.

Genes induced or repressed during CR but not during wheat rust have been highlighted in bold in Supplementary Tables 2 and 3. Several *Fp*-induced genes were induced by both pathogens, while none of the *Fp*-repressed genes were also repressed during wheat rust. The main features that stand out from this analysis are that genes classed as defence-anti-microbial and phytoalexin are primarily induced during both diseases indicating that common aspects to both defence responses exist. Genes classed as defence-ROS were primarily induced during CR and not during wheat rust suggesting that ROS may be more specifically elicited during infection by this necrotrophic pathogen.

4. Discussion

CR disease of wheat affects the base of the stem, and so characterisation of the defence responses that occur in this tissue following inoculation with *Fp* will improve our understanding of the early stages of this host–pathogen interaction. Interaction between wheat and *Fp* one day after inoculation resulted in expression changes for a range of wheat genes encoding proteins with functions associated with signal transduction and regulation of transcription that are thought to induce direct anti-microbial defences such as PR proteins that were also found to be induced. Considerable overlap was found between genes induced by *Fp* and those induced in wheat leaf tissue challenged by *P. triticina* [35], and in wheat heads following inoculation with *Fg* [29] indicating that many induced genes may form part of a general response to a wide range of pathogens.

Earlier or stronger induction of genes encoding anti-microbial proteins has been observed in resistant compared to susceptible plant varieties in other host–pathogen studies [35,54]. However, other studies have also shown that disease resistance is not always associated with induction of defence gene expression [55]. Therefore, the relevance of defence gene induction to disease resistance may vary and needs to be established for each specific host–pathogen interaction. For CR, we have previously demonstrated that defence-related *PR* gene induction occurred earlier and reached higher levels in the partially resistant cultivar Sunco compared to the susceptible Kennedy [32]. In the present study, we have identified several other genes that were induced more in Sunco than Kennedy following inoculation with *Fp*, including a gene encoding 12-OPDR that is known to be involved in the biosynthesis of jasmonate, a signalling molecule mediating defences against necrotrophic fungal pathogens [56]. Indeed, we have previously observed that MJ treatment prior to inoculation delays the development of CR symptoms in wheat [32]. This stronger induction of 12-OPDR suggests that signalling for the activation of defence responses may be greater in the partially CR-resistant cultivar Sunco, however levels of jasmonate and related molecules should be measured to test this. In contrast to defence gene induction by the pathogen, there was not a general trend of higher induction in Sunco compared to Kennedy following treatment with defence signalling chemicals. It could be speculated that differences in defence gene induction in these two cultivars may be a result of variable production of endogenous defence signalling molecules such as jasmonates, rather than variable response to them.

Many aspects of the response induced in plants following pathogen infection appear to have the potential to suppress the fungus, and yet, disease still occurs. Therefore, it is important to keep in mind that some of the changes observed may be specifically induced by pathogens as part of their infection strategy. Necrotrophic pathogens have been known to actively promote tissue senescence during infection [57–59]. *Fp*-inducible genes classed as defence-ROS were primarily not induced during wheat rust suggesting that ROS may be more specifically elicited during infection by the necrotrophic pathogen. This is consistent with the necrotrophic fungus *Fp* inducing host production of ROS to activate

programmed host cell death, whereas biotrophic pathogens such as *P. triticina* need to suppress or minimise programmed cell death. The only peroxidase reported to be highly induced by *P. triticina* was induced on average 13-fold in the resistant Thatcher-Lr34 line but less than 5-fold in the Thatcher susceptible line [35], suggesting that ROS may contribute to resistance against biotrophic pathogens in wheat. Further analysis of gene expression changes from wheat interactions with a larger number of pathogens is needed to show more conclusively how different aspects of the host response are differentially regulated in a range of host–pathogen interactions that use differing infection strategies.

Genes identified from this study that may warrant further investigation into their potential to improve disease resistance include those encoding *Fp*-inducible anti-microbial proteins. Manipulation of anti-microbial defence genes has been demonstrated to improve resistance to pathogen infection for a range of diseases [19,20,60] and a similar approach of over-expressing one or more genes encoding anti-microbial compounds may improve resistance of wheat to CR. Alternatively, manipulation of *Fp*-inducible transcription factors that often control transcription of many genes may enable activation of multiple defence genes simultaneously [16,61,62]. Down-regulation of plant defence responses that may be hijacked by *Fp* during infection, such as ROS signalling and activation of cell death, may also have the potential to improve disease development. Reduced accumulation of the mycotoxin DON during FHB caused by *Fg* has been linked to reduced symptoms and higher levels of resistance [12,13], while during CR disease DON production assists fungal colonisation [8]. Over-expression of an *Arabidopsis* UDP-glucosyltransferase has been shown to improve tolerance to DON in *Arabidopsis* [44]. Altered expression of genes encoding proteins that may metabolise DON in wheat, such as glucosyltransferases, may reduce pathogen virility. The role of signalling molecules in conferring disease resistance requires further investigation, and by altering the levels of such molecules it may be possible to improve resistance to CR, e.g. through manipulation of enzymes in the biosynthetic pathways responsible for the production of signalling compounds, such as 12-OPDR. However, jasmonates and other signalling molecules are known to be intricately involved in a number of plant stress responses, both biotic and abiotic, as well as plant development [63] therefore, extensive characterisation of transgenic lines would be required to assess performance under a range of conditions.

In conclusion, this study is the first step towards better understanding of the molecular responses triggered in wheat by fungal infection causing CR disease. Future studies may reveal how specific manipulation of the components of host defence would affect the outcome of interaction between wheat and Fp.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pmpp.2008.12.001.

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