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ORIGINAL ARTICLE

Defence gene expression profiling to *Ascochyta rabiei* aggressiveness in chickpea

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Abstract

Key message **Significant differences in defence pathway-related gene expression were observed among chickpea cultivars following *A. rabiei* infection. Differential gene expression is indicative of diverse resistances, a theoretical tool for selective breeding.**

Abstract A high number of *Ascochyta rabiei* pathotypes infecting chickpea in Australia has severely hampered efforts towards breeding for sustained quantitative resistance in chickpea. Breeding for sustained resistance will be aided by detailed knowledge of defence responses to isolates with different aggressiveness. As an initial step, the conserved and differential expressions of a suit of previously characterised genes known to be involved in fungal defence mechanisms were assessed among resistant and susceptible host genotypes following inoculation with high or low aggressive *A. rabiei* isolates. Using quantitative Real-Time PCR (qRT-PCR), 15 defence-related genes, normalised with two reference genes, were temporally differentially expressed ($P < 0.005$) as early as 2 h post inoculation of Genesis090 (resistant) or Kaniva (susceptible). The highly aggressive isolate, 09KAL09, induced vastly different expression profiles of eight key defence-related genes among resistant and susceptible genotypes. Six of these same genes were differentially expressed among ten host genotypes, inclusive of the best resistance sources within the Australian chickpea breeding program, indicating potential use for discrimination and selection of resistance "type" in future breeding pursuits.

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Introduction

Chickpea (*Cicer arietinum* L.) is an important food legume and break crop when grown in rotation with cereals and oilseeds, which ultimately improves yields and maintains soil fertility through atmospheric nitrogen fixation (Singh 1997; Dalal et al. 1998). Globally, 10.5 million tonnes of chickpea is produced annually (FAOSTAT 2013). However, the fungal pathogen *Ascochyta rabiei* (Pass.) Labr. constrains both production and quality (Nene et al. 1987; Gaur and Singh 1996). As a seed-borne pathogen, dissemination usually occurs through anthropogenic movement of seed as well as dispersal by wind and rain splash, which eventually affects all aerial plant parts (Pande et al. 2005).

Ascochyta rabiei spores germinate 12 h post inoculation (hpi) (Pandey et al. 1987). Appresoria are formed at 24 hpi and mucilaginous exudates are secreted to provide a tight contact with the host surface (Köhler et al. 1995). At this point, necrotrophic fungi are known to produce compounds, such as saponin detoxifying enzymes (Markham and Hille 2001), to suppress plant defence responses and prevent the signalling of host defence pathways (Staples and Mayer 2003). Once *A. rabiei* mycelia penetrate the

host epidermal cells (Pandey et al. 1987), they expand and secrete cell wall degrading enzymes and toxins such as solanapyrone A, B and C (Hohl et al. 1990; Alam et al. 1989; Kaur 1995). Subsequently, pycnidia are formed in the host tissue 6–8 days later to complete the life cycle (Hohl et al. 1990).

Chickpea has both active and passive defence responses to stop initial pathogenic attacks and to prevent successful invasion and spread to neighbouring cells (Coram and Pang 2006). Passive defence mechanisms include preformed structural and chemical barriers such as glandular trichomes, which secrete antifungal isoflavones (Armstrong-Cho and Gossen 2005). Active defence systems in plants may employ *R* genes to recognise pathogen-specific effectors encoded by the *Avr* genes (McDonald and Linde 2002), leading to effector-triggered immunity (ETI) and possible programmed cell death (PCD) via a hypersensitive response (HR) (Jones and Dangl 2006).

Few sources of stable resistance to *A. rabiei* exist. Singh and Reddy (1993) identified five (ICC4475, ICC6328, ICC12004, ILC200 and ILC6428) from 19,343 chickpea accessions resistant to six races of *A. rabiei* in Syria. The accession ICC3996 was added to this list by Chen et al. (2004) and together these have become the genetic basis of *A. rabiei* resistance breeding programs worldwide. However, some moderate resistance has recently broken down (Tar'an et al. 2007; Kanouni et al. 2002) and in 2010, the widely adopted resistant cultivars 'Genesis090' and 'PBA HatTrick' were heavily infected indicating that, as well as optimal environmental conditions, the pathogen may have increased aggressiveness.

Although, molecular studies of the 2010 Australian *A. rabiei* population uncovered a relatively low genetic diversity when measured with microsatellites and compared to that observed in other countries (Phan et al. 2003; Pradhan 2006; Leo et al. 2011), the low diversity for neutral genetic markers may not accurately reflect the evolutionary adaptive potential for pathogenicity of the population. Indeed, when 24 isolates with an identical microsatellite genotype were tested for their ability to cause disease on 12 Australian chickpea genotypes, extensive pathogenic variation (aggressiveness) was observed among the Australian cultivars and caused significant damage to the most current resistant cultivars (Elliott et al. 2011). This suggests that there may be differences in the perception, signalling and defence-related pathways among resistance sources to different isolates.

The defence of chickpea to *A. rabiei* is multigenic and quantitative with resistance-quantitative trait loci (R-QTL) identified on linkage groups 1, 2, 3, 4, 6 and 8 (Huettel et al. 2002; Flandez-Galvez et al. 2003; Iruela et al. 2006; Tar'an et al. 2007). This indicates that several defence-related mechanisms are involved as previously postulated (Tar'an et al. 2007). However, little is known on whether chickpea selectively employs differing types and levels of defence responses when infected with isolates of different aggressiveness known to exist in the Australian population (Elliott et al. 2011). One method to investigate this is to assess and compare, among host genotypes, the responsiveness of previously characterised defence-related genes that are representative of diverse defence-related pathways and following exposure to individual isolates of differing aggressiveness.

The expression profiles of several host genes, related to a range of defence mechanisms, have previously been characterised within the chickpea (ICC3996)—*A. rabiei* pathosystem (Coram and Pang 2005a, b, 2006). For example, pathogenesis related (PR) proteins which are induced by pathogen-derived elicitors, such as glucan and chitin within fungal cell walls, as well as fungus-secreted glycoproteins and peptides (Kombrink and Schmelzer 2001; Edereva 2005). In particular, PR-2B (β1,3-glucanase) (EC 3.2.1.39) releases glycosidic fragments that elicit host defence mechanisms, and weakens and decomposes fungal cell walls containing glucans (Kombrink and Schmelzer 2001; Edereva 2005). The speed and coordination of pathogen perception by the host is vital to achieve effective defence. Resistant hosts often respond faster and produce larger quantities of defence related compounds than susceptible ones (Yang et al. 1997). For example, PR proteins β-1,3-glucanase and chitinase (EC 3.2.1.14) are more rapidly synthesized in resistant cultivars (Vogelsang and Barz 1993; Hanselle and Barz 2001; Coram and Pang 2006; Vaghefi et al. 2013).

Meanwhile, glutathione S-transferases (GST) (EC 2.5.1.13) is a multi-gene family that protect uninfected cells from oxygen toxicity, suppress apoptosis (Coelho et al. 2010) and detoxify various compounds (Marrs 1996; Edwards et al. 2000; Dixon et al. 2002). The down-regulation of GST indicates an increase in cellular H_2O_2 from a possible oxidative burst (Neill et al. 2002). Another, Snakin-2 (SN2), is a broad-spectrum antimicrobial cysteine-rich peptide from potato (*Solanum tubersum* L.) (Segura et al. 1999), which is also known as gibberellins stimulated-like proteins (GSL2) (Meiyalaghan et al. 2014). The cysteine-rich nature of this peptide acts as both constitutive and inducible defence barriers crucial to the occurrence of disulphide bridges important in enhancing the structural stability of the plants when under stressful conditions (Berrocal-lobo et al. 2002; Pelegrini et al. 2011).

Other gene targets to asses defence to necrotrophic fungal pathogens have included the disease resistance response gene (DRRG) in pea (*Pisum sativum*) infected with *Fusarium solani* (Chiang and Hadwiger 1990) and those regulating the cellular oxidative burst in barley (*Hordeum vulgare* L.) infected with *Botrytis cinerea*. Also, members of the

NAC (for NAM, ATAF1,2 and CUC2) gene family (Peng et al. 2010), and transcription factors such as those detected in *Medicago truncatula* following infection with *Uromyces striatus* (Madrid et al. 2010). One of these, TF1082, confers an ethylene response (ER) during infection (Madrid et al. 2010). Previously, the *ERG* (ethylene receptor gene) also known as *CaETR1* (*Cicer arietinum* L. Ethylene receptor-like sequences) was found to be responsive to *A. rabiei* infection (Madrid et al. 2010). Another, TF1063, a myelobastosis (MyB) gene family member, was associated with the hypersensitive response (Madrid et al. 2010). Assessing the differential expression of these gene homologues in chickpea would aid in better understanding the complexities of defence-mechanism responses to *A. rabiei*.

High throughput quantitative Real-Time PCR (qRT-PCR) is an appropriate method to sensitively detect expression level changes of potentially low-abundance and previously characterised transcripts (Kakar et al. 2008). This approach was employed to compare the temporal and quantitative expression of key defence-related genes in chickpea to isolates that represent the breadth of aggressiveness within the Australian *A. rabiei* population. This will determine: (1) if isolates with different aggressiveness provoke differential host defence gene expressions, and the speeds in which these occur and (2) if different genes are expressed during the response, providing further evidence of different defence mechanisms among different chickpea genotypes.

Materials and methods

Plant material and fungal isolates

Ten chickpea genotypes used in this experiment (Table 1) were chosen as a representative of a differential host range based on their previously determined disease reactions to 24 Australian *A. rabiei* isolates (Elliott et al. 2011). These included susceptible and resistant cultivars which are commercially used, as well as parental lines used in the Australian breeding program. All genotypes were obtained from the Victorian Department of Environment and Primary Industries in Horsham, Victoria, Australia. Chickpea genotypes, 'Genesis090' and 'Kaniva' which were categorised as resistant (Pulse Australia 2009c) and susceptible (Carter 1999), respectively, were used to screen and selectively identify differentially expressed defence-related genes prior to testing the genes on other chickpea genotypes.

Ascochyta rabiei isolates; 09KAL09, 09MEL04, 09KAN19 and 09KIN11 used in this study were collected in 2009 (Leo et al. 2015). The isolates comprised two sets of two isolates with different aggressiveness based on the mean area under disease progress curve (AUDPC) in the pathotyping study by Elliott et al. (2011) which used the disease rating scale adopted from Singh et al. (1981). Briefly, the number of times each treatment (isolate on host) received a particular score was determined based on the established 1–9 scale. Scores of 1 & 3, 5 and 7 & 9 were grouped into three categories. For leaf infection, isolates which at 21 days post inoculation had a score of 7 or 9 greater than 80 % of the time were classified as high risk. Isolates with a score of 7 or 9 less than 60 % of the time were classified as low risk. For stem infection, isolates which at 21 days post inoculation had a score of 7 or 9 greater than 10 % of the time were classified as high risk. Isolates with a score of 7 or 9 less than 5 % of the time were classified as low risk. Overall severity/rank was based on the highest risk rating from either the stem or leaf data if they did not match. The highly aggressive isolates, 09KAL09 and 09KAN19 were isolated from the resistant cultivar, Genesis090 in Kalkee and Kaniva, Victoria, respectively. The low aggressive isolates, 09MEL04 and 09KIN11 were isolated from the moderately resistant cultivars, CICA0503 and Almaz in Melton and Kingsford, South Australia, respectively.

Bioassay

All isolates were passaged on sterilised chickpea leaves (autoclaved at 80 °C for 15 min) on 1 % (w/v) water agar for 1 week before being transferred onto V8 juice growth agar. Cultures were grown at 20 ± 2 °C with a 12 h photoperiod for 14 days. Spore suspensions were then prepared by adding 10 mL of sterile water and scraping the spores off the plate with a scalpel. The spore suspensions were

Table 1 Chickpea genotypes and disease ratings to *A. rabiei* in Australia

Genotype	Disease rating	Citations
Genesis090	Resistant (R)	Pulse Australia (2009c)
PBA HatTrick	Resistant (R)	Pulse Breeding Australia (2009)
90102-5Q-1103	Resistant (R)	Hobson (pers. comm.)
94-121*99V4006	Resistant (R)	Hobson (pers. comm.)
ICC3996	Resistant (R)	Nasir et al. (2000)
Genesis114	Moderately resistant (MR)	Pulse Australia (2009d)
Flipper	Moderately resistant (MR)	Pulse Australia (2009b)
Almaz	Moderately susceptible (MS)	Pulse Australia (2009a)
Howzat	Moderately susceptible (MS)	Pulse Australia (2009e)
Kaniva	Susceptible (S)	Carter (1999)

then filtered through a muslin cloth and the concentration adjusted to 1×10^5 spores/mL using a haemocytometer.

Seeds were surface sterilized in 5 % (w/v) sodium hypochlorite for 15 min and washed three times with sterile distilled water prior to sowing in 15 cm diameter pots (containing three seeds each) in sterile soil. A total of four biological replicates per chickpea line per isolate including mock controls was used. All plants were grown at 20 ± 4 °C for 14 days (until six to eight leaf stage) before inoculation. Plants were then sprayed with *A. rabiei* spore suspensions until run-off (approximately 5 mL/plant). Mock-inoculated controls were sprayed with sterile distilled water until run off. Following inoculation, each pot was covered with a disposable plastic cup for maximum darkness and sealed in a plastic storage box in a 20 ± 4 °C growth room to maintain humidity.

RNA extraction, cDNA preparation and development of qRT-PCR-based markers

Main stem and young leaf tissue weighed 100 mg were collected from mock and spore inoculated plants at 2, 6, 12, 24, 48 and 72 h post inoculation (hpi) for total RNA extraction using the RNeasy® Plant Mini Kit (Qiagen, CA, USA). RNase-Free DNase (Qiagen, CA, USA) was added to eliminate gDNA contamination. RNA concentration and integrity (RQI) values were determined on an Experion with RNA StdSens Chips (Bio-Rad Laboratories, CA, USA). RQI values higher than eight were used for downstream applications (Fleige and Pfaffl 2006). Total RNA (1 µg) was reverse-transcribed with a combination of $Oligo(dT)_{20}$ and random primer using the iScript™ select cDNA synthesis kit (Bio-Rad Laboratories, NSW, Australia). The quality of cDNA and absence of gDNA were assessed on agarose gel.

Seventeen genes including transcription factors which were highly and differentially expressed in various legume defence mechanisms (to mostly biotic but in some cases abiotic stress factors) were selected from the literature (Table 2). Sequences were derived from GenBank and three sets of qRT-PCR primers were designed from each using Primer3 v.0.4.0. (Rozen and Skaletsky 2000). The primers were designed with the following criteria: T_m of 60 ± 1 °C and PCR amplicon size of 55–250 bp, primer sequences length of 18–27 nucleotides and GC contents of 45–65 %. To normalise the relative quantities (NRQs) of these genes, three reference genes (PUBQ, RIB, PP2A) (Table 2) previously proven to give stable expressions after biotic stresses to *Fusarium oxysporum* f. sp. *ciceris* and *A. rabiei* in chickpea were assessed (Castro et al. 2012). All primers were synthesised at Sigma-Genosys Ltd (NSW, Australia). All primers were tested with both randomly pooled cDNA and gDNA samples, and cycle sequenced three times at the Australian Genome Research Facility (AGRF, Melbourne, Australia) to determine the correct expected amplicon size and BLASTn to ensure the amplicons were of the target sequences.

All PCR were carried out with the iQ5 Real-Time PCR detection System (Bio-Rad Laboratories, NSW, Australia). A standard curve was produced for each of the target and reference genes. The 25 µL reaction comprised 4 µL of DNA template, 13.5 µL of 1× iQSYBR Green Supermix (Bio-Rad Laboratories, NSW, Australia) and the specified primer concentration (Table 2). Thermal cycling conditions were: Initial denaturation at 95 °C for 1 min; 40 cycles of 95 °C for 10 s, 60 °C for 15 s, 72 °C for 30 s, 83 °C for 10 s (fluorescence reading), followed by melt curve analysis at 60–95 °C every 0.5 °C for 10 s. All reactions were performed in triplicate and the sample maximisation layout strategy was employed (Hellemans et al. 2007). The cDNA samples for each gene were preferably run within a single plate to reduce technical, run-to-run variation. However, inter-run calibrators (IRC) were used whenever all samples could not be analysed in the same run. Minus reverse transcription control (-RTC) and no template control (NTC) were carried out for every gene to detect the presence of contaminating DNA and/or primer dimers.

Data analysis

Data and PCR efficiency of each gene were analysed using Bio-Rad iQ5 v2.0 software (Bio-Rad, CA, USA). Reactions with more than one melt curve peak and not within the PCR efficiency range of 95–110 % were discarded.

Reference genes were analysed and selected based on stable expression using geNorm^{PLUS} (Hellemans et al. 2007). Normalization of expression values from targeted genes were calculated using qbase PLUS software, and were reported as normalized relative quantities (NRQs) (Hellemans et al. 2007).

General linear model was performed using SAS and Minitab 16 to determine differentially expressed genes at $P < 0.05$. A mean fold change of 2.0 was used as the cut-off point. Differentially expressed genes between genotypes, treatments, or genotype × treatment interactions, were clustered using an hierarchical cluster analysis. A data matrix for each genotype with the expression ratio was used to calculate an Euclidean distance matrix. The UPMG method was used to generate a dendogram using *K-means* clustering with Cluster v3.0 (Eisen et al. 1998) and viewed with Treeview v1.60 (Page 1996) as a heat map.

To assess for differences in host gene expression levels when infected with different isolates, the mean expressions derived from each interaction were compared and an analysis of variance (ANOVA) was then performed with qbase PLUS software ($P = 0.05$). The same analyses determined significant gene expression differences among ten chickpea

Table 2 Novel and published genes and primers used for differential gene expressions in chickpea genotypes

Gene ID	Gene name (abbreviation)	Biological significance	Primers	Primer conc (μM)	Product size (bp)	References
CV793598	Pathogenesis-related protein 2B (PR2B)	β-1,3-Glucanase—hydrolysis of flavonoid and isoflavonoid compounds	F: GCCTAGAAAGGCAAATCCTTC R: CATCTGCCGTGG-GAATAAGA	0.15	153	Coram and Pang (2006)
DY475248	Polymorphic antigen membrane protein (PAMP)	Transcription of defence-related genes—resistance response via metabolism utilising polyamines (PA) and nicotianamines (NAs)	F: CCGCTGATACAGTGGAGGTT R: GTTTCCCCAATTTCCTCACC	0.30	166	
DY475250	Glutathione *S*-transferase (GST)	Regulation of host cellular H_2O_2	F: TCCCTCCAACCTACTAACAAGG R: TTTGGATTGGATAAGATTTGGTTT	0.30	119	
CV793608	SNAKIN2 antimicrobial peptide precursor (SN2)	Regulate the production of reactive oxygen species (ROS) in host and enhance host structural ability through disulphide bridges	F: CATGGCAACAAGACCAAGTGTC R: GTTGGGAACAAAGTAGGGACTG	0.30	102	
DY475397	Superoxide dismutase copper chaperone precursor (SDCCP)	Detoxification of reactive superoxide radical anions produced by fungal pathogen	F: TCTCACTCTCACCAATCCCTAAA R: CCACCATAAACTCCGTCAGTAAC	0.30	187	
CV793599	Protein with leucine-zipper (LZP)	bZIP transcription factors regulating salicylic acid (SA)	F: AAGACATTGCATTGCAGCAG R: AAGACAAGGCTTTGCTCCAA	0.30	176	
DY396298	Environmental stress-inducible protein (ESP)	Environmental stress induced protein	F: CGGGAATTCGATTAAGCAGT R: ACCGTTGTAACCACCTCCAC	0.15	178	
TF 1070.m00005	Myb, DNA-binding, Homeodomain like (TF1063)	Host defence gene (QTL_{AR1}–LG4)	F: GTTATGTGGGTGGAGTTGGAA R: CAACCATAGCTGCAACCATCT	0.15	104	Madrid et al. (2010)
TC101530	Pathogenesis-related transcriptional factor (TF1082)	Host defence gene (QTL_{AR2}–LG4)	F: AAGTCTTATCGTGGCGTTCG R: TCATAAGCTAGTGCTGCTGCT	0.15	131	

Table 2 continued

Gene ID	Gene name (abbreviation)	Biological significance	Primers	Primer conc (μM)	Product size (bp)	References
CR955005	Resistance gene analog 4—LG8 (RGA 4)	Chickpea RGA families linked to NBS-LRR genes	F: GGCCATTGAATCAAGA CGAG R: CACATTTCA CCACAATCTCC	0.30	113	Palomino et al. (2009)
DY396288	Resistance gene analog 5- LG2 (RGA 5)		F: GAACGACGACCAAG ATAC R: CCATTTACGACTTCC GCAC	0.30	140	
AW774607	Resistance gene analog 7—LG3 (RGA 7)		F: GCGACCGTCTTGTA TGACAC R: GGAGCTTCCTGTT GTATAGCC	0.30	211	
CX533869	Resistance gene analog 10—LG6 (RGA 10)		F: TGCCGTATTGCTG ATCTGA R: TAGATGCGTTGTGA AGATT	0.30	124	
EU 339183	*CarNAC* (CARNAC)	Developmental process and plant defense	F: CTCTTTCCCTTTACCCG R: TTGGCTTCTTTAGTGCTG	0.30	243	Peng et al. (2010)
DY396400	*CaETR1* (*Cicer arietinum* L. Ethylene receptor-like sequences)	Ethylene response—induced transcription factor found on QTL_{AR1} activated against *A. rabiei* pathotype II	F: TAGGGTTTGGACCAAG CAAG R: CTTCTGAGACTGCT GCAACG	0.30	151	Madrid et al. (2012)
AJ515032	Polyubiquitin (PUBQ)	House keeping gene	F: AGGTGGAAAGTTC AGACACAAT R: ACCTTTGCTGATCT GGTGGGA	0.30	80	Castro et al. (2012)
AJ131050.1	Ribulose 1,5-biphosphate carboxylase small subunit (RIB)	House keeping gene	F: CCACCATTGACTGA AGAGCA R: TTGAACAGCCTCAGT GCAAC	0.30	192	

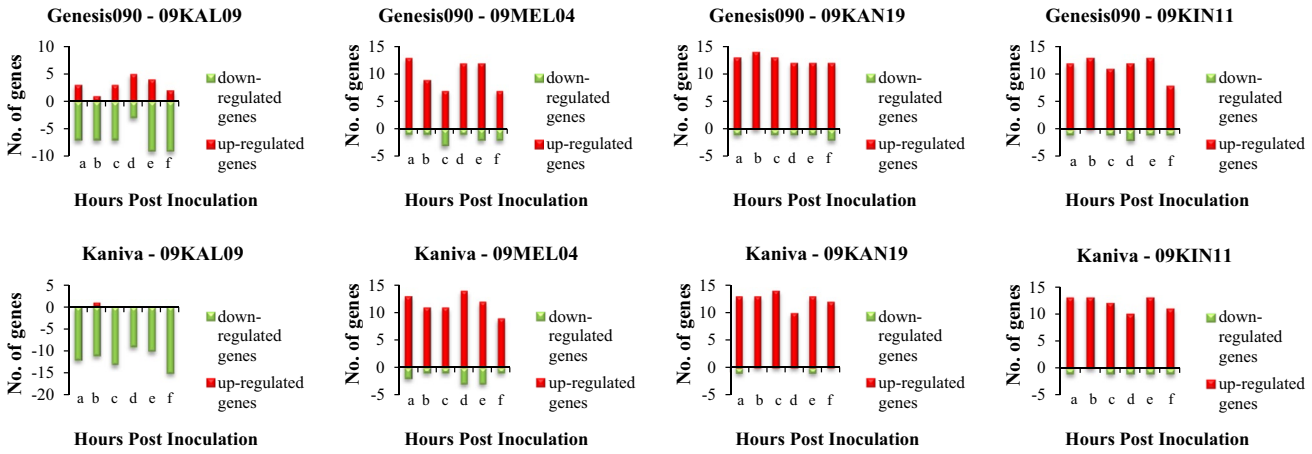

Fig. 1 Kinetic trends of differential gene expression for Genesis090 (resistant) and Kaniva (susceptible) genotypes over the time course of infection: **a** 2 hpi; **b** 6 hpi; **c** 12 hpi; **d** 24 hpi; **e** 48 hpi; **f** 72 hpi, with 4 *A. rabiei* isolates, 09KAL09, 09MEL04, 09KAN19 and 09KIN11. The *vertical axis* indicates the number of up-regulated (*red*) and down-regulated genes (*green*) at each time point

genotypes (Table 1) infected with the aggressive isolate, 09KAL09. A total of eight defence genes which were up-regulated in 'Genesis090' when infected with 09KAL09 (CARNAC, GST, PR2B, SN2, ERG, PAMP, RGA4, TF1082) were selected to identify and determine potential responses induced among four other resistant, two moderately resistant and two moderately susceptible chickpea genotypes relative to the susceptible genotypes (Table 1) and compared using ANOVA.

Results

Single fragments of 80–250 bp were amplified with efficiencies of 90–110 % from 15 of the 17 target genes and were used to assess expression levels with qRT-PCR (Table 2). Of the sequences tested for suitability for expression normalisation, PUBQ and RIB were the most stable with *M* (gene stability) values of 1.102 and coefficients of variation (CV) values of 0.414 and 0.412, respectively (Hellemans et al. 2007). The *M* value for PP2A was 1.411 and thus excluded.

Timing and expression levels of defence-related host genes based on interactions with different levels of isolate aggressiveness

All 15 defence-related genes were differentially expressed in at least one time point following inoculation of 'Genesis090' and 'Kaniva' when compared to the un-inoculated controls. Of the four isolates assessed, the highly aggressive 09KAL09, produced a grossly different expression profile across all 15 genes. The most down-regulation, was consistent in timing and levels across both the resistant (Genesis090) and susceptible (Kaniva) genotypes (Fig. 1). Conversely, the expression profile produced by the other highly aggressive isolate (09KAN19) was not largely different in either timing or magnitude to the two less aggressive isolates (09MEL04 and 09KIN11). Again, this was consistent among host genotypes (Figs. 1, 2). In general, the majority of differentially expressed genes were up-regulated as early as 2 hpi and started to be down-regulated at 72 hpi (Supplementary Material 1).

Similarities in host gene expression trends among isolate interactions

Cluster analysis shown similarities in individual gene expression profiles following exposure to each of the four isolates (Fig. 2). Mega Cluster I contained PR2B, up-regulated as early as 12 hpi in 'Kaniva' and 'Genesis090' regardless of isolate applied. Cluster II was divided into six subgroups. Cluster II.1:2 comprised ERG and LZP genes, which were not up-regulated when either host genotype was inoculated with either 09KAL09 or 09MEL04, but were up-regulated when inoculated with 09KAN19 or 09KIN11 (as early as 2 hpi). Cluster II.2:3 comprised CARNAC, GST and SDCCP. CARNAC was up-regulated in all interactions except when Kaniva was inoculated with 09KAL09, suggesting that lack of expression may lead to susceptibility when inoculated with the highly aggressive isolates. GST and SDCCP were up-regulated in at least one time point in both genotypes when exposed to any of the isolates. Cluster II.3:5 comprised PAMP, RGA4, RGA7, TF1063 and SPK. Cluster II.4:1 contained TF1082, Cluster II.5:2 and Cluster II.6:1 comprised RGA10&5, and SN2, respectively. Genes in Cluster II 3, 4, 5 and 6 were up-regulated in both genotypes infected with 09KAN19 and

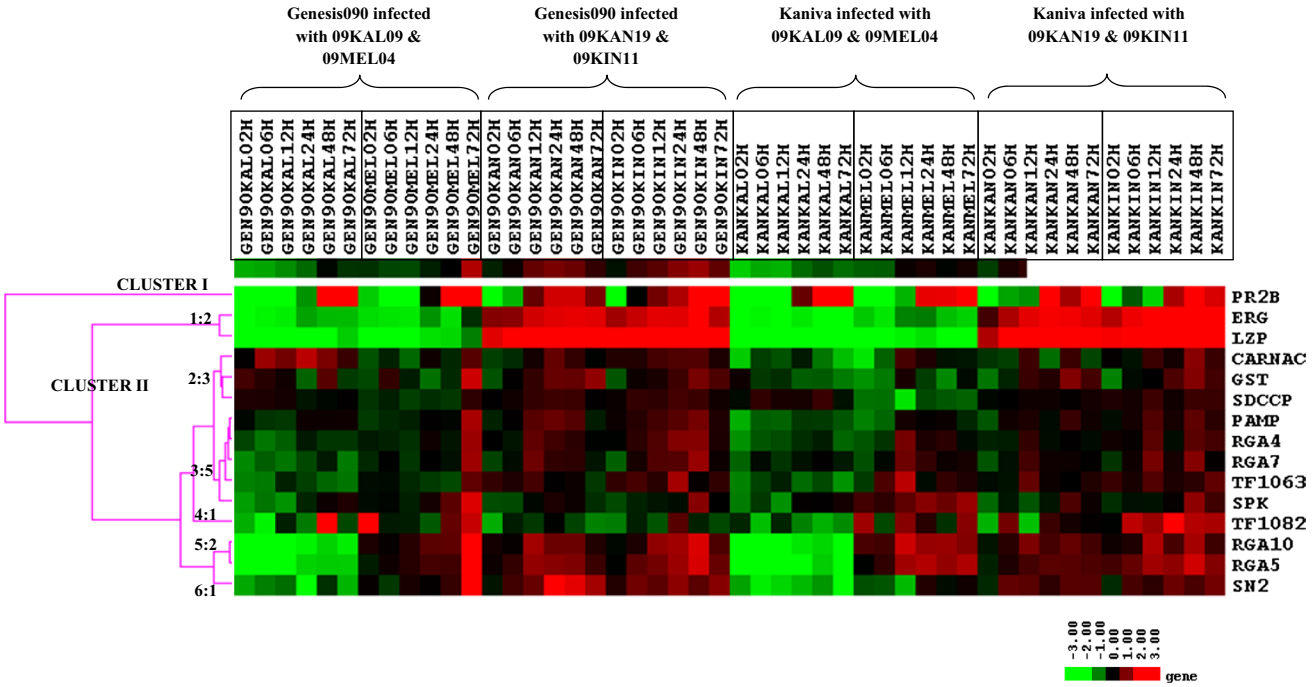

Fig. 2 Heatmap of 15 genes expression profiles for Genesis090 (resistant) and Kaniva (susceptible) over the time course after infection with 4 *A. rabiei* isolates, 09KAL09, 09MEL04, 09KAN19 and 09KIN11. Up-regulation is indicated in *red*, down-regulation is indicated in *green*, normalised expression values close to the mean are in *black*. No detectable expression is in *grey*. The Log_2 values of the expression profile for each treatment and genotype were normalised with two reference genes and non-inoculates samples

09KIN11 as early as 6 hpi. When infected with 09MEL04, these genes were up-regulated at later infection stages (>12 hpi), but remained at either a basal expression rate or were down-regulated when the genotypes were infected with 09KAL09.

Differentially expressed defence-related genes and their relations to different levels of host susceptibility

Following inoculation with isolate 09KAL09, major differences in the gene differential expression profiles were observed among the ten host genotypes assessed, which ranged in classification from resistant to susceptible (Supplementary material 2; Fig. 3). The mean expression profiles of each genotype at a 95 % confidence interval identified six genes, CARNAC, ERG, GST, RGA4, SN2 and TF1082, differentially expressed across all ten host genotypes (Supplementary material 2). The expression of PR2B was not differential but consistently highly up-regulated among all hosts. PAMP was up-regulated in all hosts except for the highly susceptible Kaniva, perhaps indicating a lack of recognition. To further identify which genes were up-regulated in the resistant and moderately resistant genotypes in comparison to the susceptible genotypes, the mean expression profiles of each genotype were categorised into their susceptibility levels and compared (Supplementary materials 3, 4). Between moderately resistant and resistant genotypes, only SN2 was differentially expressed, more highly in moderately resistant genotypes. Interestingly, no genes were differentially expressed between moderately resistant and moderately susceptible classified genotypes at any of the time points assessed, however, four genes, SN2, GST, ERG and RGA4, were differentially expressed between resistant/moderately resistant and susceptible genotypes. Three, SN2, GST, and ERG, were expressed at higher levels in resistant/moderately resistant than susceptible genotypes and one, RGA4, at a higher level in susceptible genotypes. Following validation across a broader germplasm and in response to a larger number of isolates, the differential expression of these four genes may be useful as tools for future molecular selection of resistance within breeding programs.

Discussion

For the first time, they study has demonstrated that *A. rabiei* isolates of a similar high aggressiveness level are able to cause different host responses within the same chickpea genotype. One might postulate that 09KAL09 is able to evade detection and recognition and then goes on to suppress host defence responses whilst it establishes itself

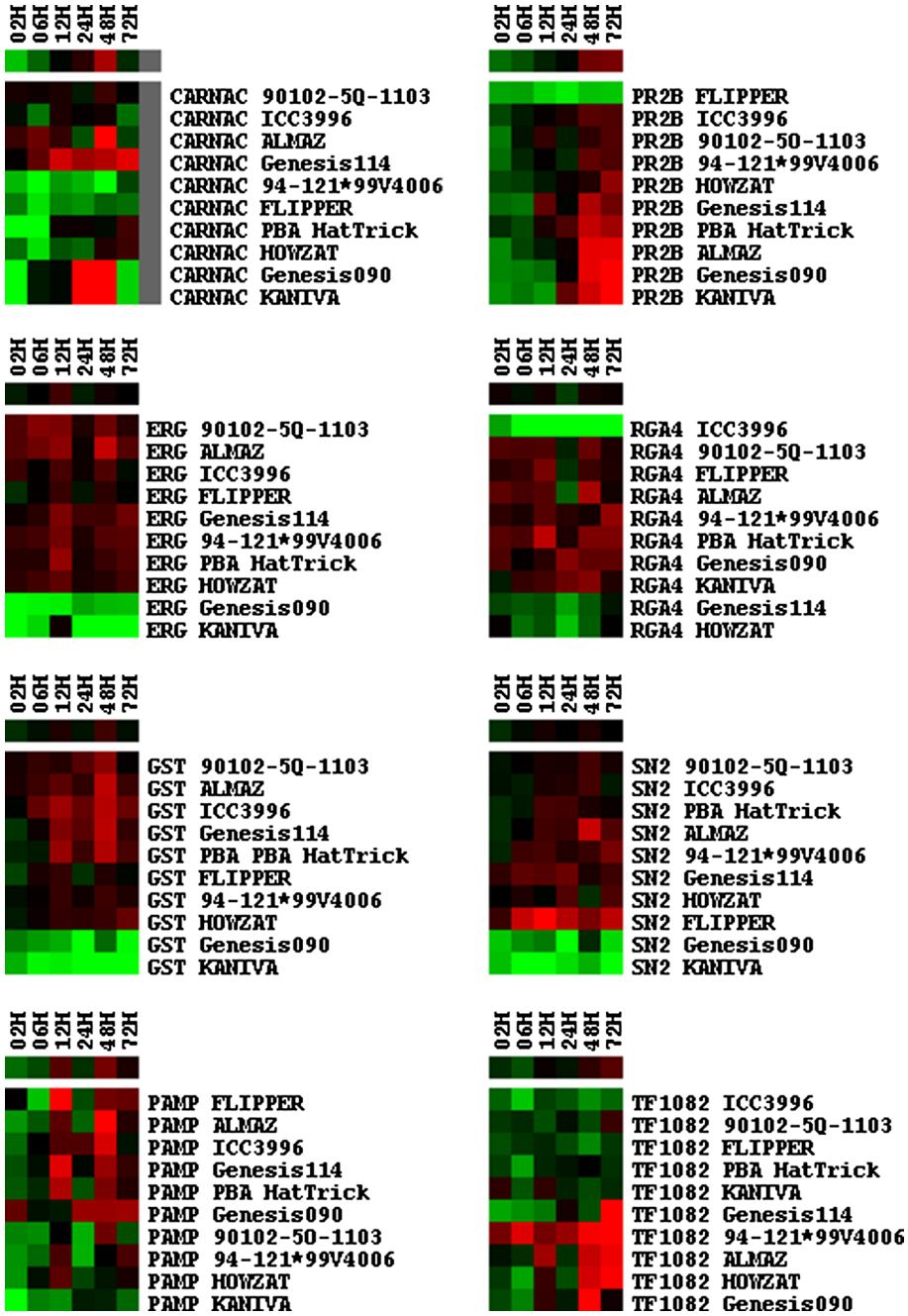

Fig. 3 Heatmap of eight genes expression profiles for 10 chickpea genotypes (Table 1) over the time course after infection with the most pathogenic *A. rabiei* isolate, 09KAL09. Up-regulation is indicated in *red*, down-regulation is indicated in *green*, normalised expression values close to the mean are in *black*, no detectable expression is in *grey*. The Log_2 values of the expression profile for each treatment and genotype were normalised with two reference genes and non-inoculates samples

and begins to evade and colonise the tissues. Meanwhile 09KAN19, also highly aggressive, is detected and recognised almost immediately (and certainly by 2 hpi), causing the up-regulation of the spectrum of defence responses related to the genes under study. The question remains regarding what differentiates the ability for the 09KAL09 isolate to be highly aggressive compared to the two less aggressive isolates. Perhaps other isolate-related fitness characteristics are important in establishing and maintaining infection ahead of host defences? Certainly, the timing of gene expressions was largely indifferent following exposure to the highly aggressive 09KAN19 or either of the less aggressive isolates, indicating that molecular evidence of pathogenicity differences among these three isolates was not captured in this study and on these cultivars, hence a wider range of defence-related genes and cultivars would need to be assessed. This would be more feasible with whole genome transcriptomics in response to *A. rabiei* inoculation.

Another plausible reason to the down regulation of most differentially expressed genes in both susceptible and resistant genotypes is the production, deletion or selection

of fungal effectors in 09KAL09 that impact on pathogen recognition. This may trigger different host defence mechanisms. Positive selection occurring within the effector proteins has been observed quite extensively for *Phytophtora sojae* of soybean (Jiang et al. 2008). This was postulated as a mechanism employed to enable escape from host resistance protein detection and potentially adapt to different host virulence targets (Ellis et al. 2009). Indeed, mutation of motifs in the C-terminus of an Avr1b protein reduced the ability of the pathogen to suppress programmed cell death (PCD) and also abolished the avirulence interaction of Avr1b with the *Rps1b* resistance gene in soybean (Dou et al. 2008). A similar mechanism may be occurring within *A. rabiei* isolate 09KAL09, enabling it to evade detection by chickpea.

During industry establishment, selection of a narrow gene pool and subsequent inbreeding has led to a lack of genome diversity across cultivated chickpea, which has also likely constricted the potential diversity of defence mechanisms retained within Australian chickpea cultivars. This low diversity of defence mechanisms was shown in the considerably fewer number of disease resistance gene homologues in chickpea in comparison to other legume species (Varshney et al. 2013). However, significant differences in expression levels and timings of the 15 defence-related genes assessed in the current study were detected among the 10 host genotypes assessed. At very early time points (2–6 hpi) these are likely related to differences in the timing of pathogen recognition and subsequent speed to signal down-stream defence mechanisms.

Indeed, the faster expression of GST in 'Kaniva' (6 hpi) compared to 'Genesis090' (24 hpi) is likely associated with the earlier accumulation of H_2O_2 in the susceptible cultivar to trigger a rapid hypersensitive response. However, across genotypes, the susceptible genotypes produced significantly less GST than the resistant ones at earlier time points and greater expression in the resistant genotypes later on (24–48 hpi). This may indicate that although the hypersensitive response is employed by susceptible genotypes this is not effectual for containing the pathogen and that resistant genotypes only instigate this defence response after other first-line defence responses have been triggered. Indeed, other reactive oxygen species involved in the precursors to the hypersensitive response have been detected in resistant genotypes at earlier time points of the interaction (Hohl et al. 1990; Coram and Pang 2006).

The pattern of expression of SN2 was similar to GST (another antioxidant) with greater quantities detected in resistant/moderately resistant genotypes than susceptible genotypes. This is in accordance to the up-regulation of SN2 previously detected in ICC3996 (Coram and Pang 2005b, 2006). Sequence similarities of SN2 peptides to GIP2 (GASA-like protein) from *Petunia hybrida* suggests involvement in redox regulations which regulate the production of reactive oxygen species in pathogenesis and wounding (Berrocal-lobo et al. 2002; Wigoda et al. 2006; Balaji and Smart 2012).

Meanwhile, *CaETR1* (*Cicer arietinum* L. ethylene receptor-like sequences) was the first ethylene receptor discovered in chickpea associated with *A. rabiei* resistance (Madrid et al. 2010). The ERG locus is closely linked to a major QTL, QTL_{AR1} proposed to condition resistance to pathotype II (Iruela et al. 2006; Madrid et al. 2012). Recently, the *CaETR1* and *CaETR-1a*/*CaETR-1b* alleles from resistant and susceptible chickpea genotypes (Madrid et al. 2012) were used to negatively select and eliminate susceptible individuals from a breeding program (Madrid et al. 2013). The differential expression of the allele (unknown) observed in the current study between resistant/moderately resistant and susceptible genotypes may further indicate its suitability for resistance selection across broad range of germplasm.

A spectrum of differences in levels and timings of the CARNAC transcription factor was observed among the 10 genotypes. Down- or unaltered expression in the majority of genotypes may be related to involvement in developmental processes such as apical meristem development, flowering and secondary wall formation (Peng et al. 2010). This may be anticipated in response to a pathogen attack, as reserves are rerouted to defence-related activities (Coram and Pang 2006). However, up-regulation witnessed in Almaz, Genesis114, Genesis090 and Kaniva may have been directly related to defence responses through participation in signalling pathways and regulatory networks (Nuruzzaman et al. 2013). Indeed, NAC proteins activate PR genes, induce the hypersensitive response and cause cell death at the infection site (Kaneda et al. 2009; Seo et al. 2010). NAC proteins also have the ability to form alliances with certain host regulatory complexes, enabling them to act as negative regulators of the defence response by suppressing defence-related genes (Wang et al. 2009).

Meanwhile, PR proteins are pathogen-induced proteins classified into 17 families from PR-1 to PR-17, based on biochemical properties (Van Loon et al. 2006). As observed for PR2B in the current study, they may be expressed prior to infection due to involvement in plant development (Edereva 2005), accumulated and synthesized for a long lag period. They may then be translocated from the site of induction to other plant parts during pathogenic attack (Matsuoka and Ohashi 1986). Ultimately, PR2B (β1,3-glucanase) produces glycosidic fragments which weakens and decomposes fungal cell walls containing glucans, chitin and proteins (Kombrink and Schmelzer 2001; Edereva 2005). As previously reported, the PR2B gene was significantly up-regulated at 48–72 hpi compared to other time points, particularly in 'Genesis114', 'PBA HatTrick',

'Almaz', 'Genesis090' and 'Kaniva' (Hanselle and Barz 2001; Coram and Pang 2006; Cho and Muehlbauer 2004).

The gene deemed to regulate polymorphic antigen proteins (PAMP) was up-regulated at 12 and 48 hpi in all genotypes except for the susceptible 'Kaniva'. This gene is likely to be a homologue of Enolase phosphatise E1 protein, a bifunctional enzyme of methionine salvage that regenerates methionine from 5′-methylthioadenosine (MTA) (Wang et al. 2005). Its function in the resistance response is likely via its metabolism that utilises polyamines (PAs), nicotianamines (NAs) and interacts with ethylene biosysnthesis (Waduwara-Jayabahu et al. 2012). PAs are associated with cell division as a response to abiotic and biotic stress (Takahashi and Kakehi 2010; Vera-Sirera et al. 2010). NAs act as chelators for long distance ion transport and defence signalling processes (Curie et al. 2009), and ethylene is a phytohormone capable of signalling within defence pathways.

The transcription factor TF1082 was increasingly upregulated in several resistant and moderately resistant genotypes from 48 hpi onwards after exposure to the highly aggressive isolate 09KAL09. Previously, in *Medicago truncatula* infected with *Uromyces striatus*, this gene was up-regulated in resistant genotypes and down-regulated in susceptible genotypes, thought to bind to the GCC box of PR gene promoters and confer ethylene responsiveness (Madrid et al. 2010).

In conclusion, this study showed that chickpea has a number of defence-related mechanisms which are activated simultaneously to mount defence to *A. rabiei*, confirming that it is a race-nonspecific resistance controlled by genes with minor to intermediate and additive effects. Although a small subset of genes was assessed, several were differentially expressed among cultivars, further indicating the potential of different defence mechanisms in chickpea under controlled conditions where all plants are subjected to the same environment conditions. Further studies such as RNA sequencing and identifying sequence polymorphisms of within or upstream or downstream signalling regions of the differentially expressed genes in susceptible and resistant cultivars may identify potential allelic differences that, once functionally validated, could be converted into stable markers for future selective breeding purposes. Breeding chickpea genotypes containing several defence strategies will improve durability against the pathogenic diversity of the pathogen population.

Author contribution statement AEL participated in the design of the experiment, carried out the experimental work, performed the statistical analyses, and drafted the manuscript. CCL assisted in revising the manuscript. RF participated in the design of the experiment and drafted the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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