1 **Title:** Defence gene expression profiling to *Ascochyta rabiei* aggressiveness in chickpea.

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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), fifteen defence-related genes, normalised with two reference genes, were temporally differentially expressed ($P < 0.005$) as early as 2 hours 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.

33 **Keywords: *Ascochyta rabiei*, chickpea, plant-pathogen interaction, molecular defence**
34 **mechanisms**

35

36 ## Introduction

37 Chickpea (*Cicer arietinum* L.) is an important food legume and break crop when grown in
38 rotation with cereals and oilseeds, which ultimately improves yields and maintains soil
39 fertility through atmospheric nitrogen fixation (Singh 1997; Dalal et al. 1998). Globally, 10.5
40 million tonnes of chickpea is produced annually (FAOSTAT 2013). However, the fungal
41 pathogen *Ascochyta rabiei* (Pass.) Labr. constrains both production and quality (Nene et al.
42 1987; Gaur and Singh 1996). As a seed-borne pathogen, dissemination usually occurs
43 through anthropogenic movement of seed as well as dispersal by wind and rain splash, which
44 eventually affects all aerial plant parts (Pande et al. 2005).

45 *A. rabiei* spores germinate 12 hours post inoculation (hpi) (Pandey et al. 1987). Appresoria
46 are formed at 24 hpi and mucilaginous exudates are secreted to provide a tight contact with
47 the host surface (Köhler et al. 1995). At this point, necrotrophic fungi are known to produce
48 compounds, such as saponin detoxifying enzymes (Markham and Hille 2001), to suppress
49 plant defence responses and prevent the signalling of host defence pathways (Staples and
50 Mayer 2003). Once *A. rabiei* mycelia penetrate the host epidermal cells (Pandey et al. 1987),
51 they expand and secrete cell wall degrading enzymes and toxins such as solanapyrone A, B
52 and C (Hohl et al. 1990; Alam et al. 1989; Kaur 1995). Subsequently, pycnidia are formed in
53 the host tissue 6 to 8 days later to complete the life cycle (Hohl et al. 1990).

54 Chickpea has both active and passive defence responses to stop initial pathogenic attacks and
55 to prevent successful invasion and spread to neighbouring cells (Coram and Pang 2006).

56 Passive defence mechanisms include preformed structural and chemical barriers such as
57 glandular trichomes, which secrete antifungal isoflavones (Armstrong-Cho and Gossen
58 2005). Active defence systems in plants may employ *R* genes to recognise pathogen-specific
59 effectors encoded by the *Avr* genes (McDonald and Linde 2002), leading to effector-triggered
60 immunity (ETI) and possible programmed cell death (PCD) via a hypersensitive response
61 (HR) (Jones and Dangl 2006).

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

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

80 The defence of chickpea to *A. rabiei* is multigenic and quantitative with resistance-
81 quantitative trait loci (R-QTL) identified on linkage groups 1, 2, 3, 4, 6 and 8 (Huettel et al.
82 2002; Flandez-Galvez et al. 2003; Iruela et al. 2006; Tar'an et al. 2007). This indicates that
83 several defence-related mechanisms are involved as previously postulated (Tar'an et al.
84 2007). However, little is known on whether chickpea selectively employs differing types and
85 levels of defence responses when infected with isolates of different aggressiveness known to
86 exist in the Australian population (Elliott et al. 2011). One method to investigate this is to
87 assess and compare, among host genotypes, the responsiveness of previously characterised
88 defence-related genes that are representative of diverse defence-related pathways and
89 following exposure to individual isolates of differing aggressiveness.

90 The expression profiles of several host genes, related to a range of defence mechanisms, have
91 previously been characterised within the chickpea (ICC3996) - *A. rabiei* pathosystem (Coram
92 and Pang 2005a; Coram and Pang 2005b; Coram and Pang 2006). For example, Pathogenesis
93 Related (PR) proteins which are induced by pathogen-derived elicitors, such as glucan and
94 chitin within fungal cell walls, as well as fungus-secreted glycoproteins and peptides
95 (Kombrink and Schmelzer 2001; Edereva 2005). In particular, PR-2B (β1, 3-glucanase) (EC
96 3.2.1.39) releases glycosidic fragments that elicit host defence mechanisms, and weakens and
97 decomposes fungal cell walls containing glucans (Kombrink and Schmelzer 2001; Edereva
98 2005). The speed and coordination of pathogen perception by the host is vital to achieve
99 effective defence. Resistant hosts often respond faster and produce larger quantities of
100 defence related compounds than susceptible ones (Yang et al. 1997). For example, PR
101 proteins β-1,3-glucanase and chitinase (EC 3.2.1.14) are more rapidly synthesized in resistant
102 cultivars (Volgesang and Barz 1993; Hanselle and Barz 2001; Coram and Pang 2006;
103 Vaghefi et al. 2013).

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

114 Other gene targets to asses defence to necrotrophic fungal pathogens have included the
115 disease resistance response gene (DRRG) in pea (*Pisum sativum*) infected with *Fusarium*
116 *solani* (Chiang and Hadwiger 1990) and those regulating the cellular oxidative burst in barley
117 (*Hordeum vulgare* L,) infected with *Botrytis cinerea*. Also, members of the NAC (for NAM,
118 ATAF1,2 and CUC2) gene family (Peng et al. 2010), and transcription factors such as those
119 detected in *Medicago truncatula* following infection with *Uromyces striatus* (Madrid et al.
120 2010). One of these, TF1082, confers an ethylene response (ER) during infection (Madrid et
121 al. 2010). Previously, the *ERG* (ethylene receptor gene) also known as *CaETR1* (*Cicer*
122 *arietinum* L. Ethylene receptor-like sequences) was found to be responsive to *A. rabiei*
123 infection (Madrid et al 2010). Another, TF1063, a myelobastosis (MyB) gene family
124 member, was associated with the hypersensitive response (Madrid et al. 2010). Assessing the
125 differential expression of these gene homologues in chickpea would aid in better
126 understanding the complexities of defence-mechanism responses to *A. rabiei*.

127 High throughput quantitative Real-Time PCR (qRT-PCR) is an appropriate method to
128 sensitively detect expression level changes of potentially low-abundance and previously

129 characterised transcripts (Kakar et al. 2008). This approach was employed to compare the
130 temporal and quantitative expression of key defence-related genes in chickpea to isolates that
131 represent the breadth of aggressiveness within the Australian *A. rabiei* population. This will
132 determine: 1) if isolates with different aggressiveness provoke differential host defence gene
133 expressions, and the speeds in which these occur and 2) if different genes are expressed
134 during the response, providing further evidence of different defence mechanisms among
135 different chickpea genotypes.

136

137 **Material and methods**

138 ***Plant material and fungal isolates***

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

149 *A. rabiei* isolates; 09KAL09, 09MEL04, 09KAN19 and 09KIN11 used in this study were
150 collected in 2009 (Leo et al. 2015). The isolates comprised two sets of two isolates with
151 different aggressiveness based on the mean area under disease progress curve (AUDPC) in

152 the pathotyping study by Elliott et al. (2011) which used the disease rating scale adopted from
153 Singh et al. (1981). Briefly, The number of times each treatment (isolate on host) received a
154 particular score was determined based on the established 1-9 scale. Scores of 1 & 3, 5 and 7
155 & 9 were grouped into three categories. For leaf infection, isolates which at 21 days post
156 inoculation had a score of 7 or 9 greater than 80 % of the time were classified as high risk.
157 Isolates with a score of 7 or 9 less than 60 % of the time were classified as low risk. For stem
158 infection, isolates which at 21 days post inoculation had a score of 7 or 9 greater than 10 % of
159 the time were classified as high risk. Isolates with a score of 7 or 9 less than 5 % of the time
160 were classified as low risk. Overall severity/rank was based on the highest risk rating from
161 either the stem or leaf data if they did not match. The highly aggressive isolates, 09KAL09
162 and 09KAN19 were isolated from the resistant cultivar, Genesis090 in Kalkee and Kaniva,
163 Victoria, respectively. The low aggressive isolates, 09MEL04 and 09KIN11 were isolated
164 from the moderately resistant cultivars, CICA0503 and Almaz in Melton and Kingsford,
165 South Australia, respectively.

166

167 ***Bioassay***

168 All isolates were passaged on sterilised chickpea leaves (autoclaved at 80 °C for 15 mins) on
169 1 % (w/v) water agar for 1 week before being transferred onto V8 juice growth agar. Cultures
170 were grown at 20 ± 2 °C with a 12 h photoperiod for 14 days. Spore suspensions were then
171 prepared by adding 10 mL of sterile water and scraping the spores off the plate with a scalpel.
172 The spore suspensions were then filtered through a muslin cloth and the concentration
173 adjusted to $1x10^5$ spores/mL using a haemocytometer.

174 Seeds were surface sterilized in 5 % (w/v) sodium hypochlorite for 15 minutes and washed
175 three times with sterile distilled water prior to sowing in 15 cm diameter pots (containing 3

176 seeds each) in sterile soil. A total of four biological replicates per chickpea line per isolate
177 including mock controls was used. All plants were grown at 20 ± 4 °C for 14 days (until six
178 to eight leaf stage) before inoculation. Plants were then sprayed with *A. rabiei* spore
179 suspensions until run-off (approximately 5 mL/plant). Mock-inoculated controls were
180 sprayed with sterile distilled water until run off. Following inoculation, each pot was covered
181 with a disposable plastic cup for maximum darkness and sealed in a plastic storage box in a
182 20 ± 4 °C growth room to maintain humidity.

183

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

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

195 Seventeen genes including transcription factors which were highly and differentially
196 expressed in various legume defence mechanisms (to mostly biotic but in some cases abiotic
197 stress factors) were selected from the literature (Table 2). Sequences were derived from
198 GenBank and three sets of qRT-PCR primers were designed from each using Primer3 v.0.4.0.
199 (Rozen and Skaletsky 2000). The primers were designed with the following criteria: T_m of 60

200 ± 1°C and PCR amplicon size of 55-250 bp, primer sequences length of 18–27 nucleotides
201 and GC contents of 45–65 %. To normalise the relative quantities (NRQs) of these genes,
202 three reference genes (PUBQ, RIB, PP2A) (Table 2) previously proven to give stable
203 expressions after biotic stresses to *Fusarium oxysporum* f. sp. *ciceris* and *A. rabiei* in
204 chickpea were assessed (Castro et al. 2012). All primers were synthesised at Sigma-Genosys
205 Ltd (NSW, Australia). All primers were tested with both randomly pooled cDNA and gDNA
206 samples, and cycle sequenced three times at the Australian Genome Research Facility
207 (AGRF, Melbourne, Australia) to determine the correct expected amplicon size and BLASTn
208 to ensure the amplicons were of the target sequences.

209 All PCR were carried out with the iQ5 Real-Time PCR detection System (Bio-Rad
210 Laboratories, NSW, Australia). A standard curve was produced for each of the target and
211 reference genes. The 25 µL reaction comprised 4 µL of DNA template, 13.5 µL of 1X
212 iQSYBR Green Supermix (Bio-Rad Laboratories, NSW, Australia) and the specified primer
213 concentration (Table 2). Thermal cycling conditions were: Initial denaturation at 95 °C for 1
214 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
215 reading), followed by melt curve analysis at 60-95°C every 0.5°C for 10 s. All reactions were
216 performed in triplicate and the sample maximisation layout strategy was employed
217 (Hellemans et al. 2007). The cDNA samples for each gene were preferably run within a
218 single plate to reduce technical, run-to-run variation. However, inter-run calibrators (IRC)
219 were used whenever all samples could not be analysed in the same run. Minus reverse
220 transcription control (-RTC) and no template control (NTC) were carried out for every gene
221 to detect the presence of contaminating DNA and/or primer dimers.

222

223 ***Data analysis***

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

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

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

238 To assess for differences in host gene expression levels when infected with different isolates,
239 the mean expressions derived from each interaction were compared and an analysis of
240 variance (ANOVA) was then performed with qbase PLUS software (P =0.05). The same
241 analyses determined significant gene expression differences among ten chickpea genotypes
242 (Table 1) infected with the aggressive isolate, 09KAL09. A total of eight defence genes
243 which were up-regulated in 'Genesis090' when infected with 09KAL09 (CARNAC, GST,
244 PR2B, SN2, ERG, PAMP, RGA4, TF1082) were selected to identify and determine potential
245 responses induced among four other resistant, two moderately resistant and two moderately
246 susceptible chickpea genotypes relative to the susceptible genotypes (Table 1) and compared
247 using ANOVA.

248

249 ## Results

250 Single fragments of 80 to 250 bp were amplified with efficiencies of 90 to 110 % from 15 of
251 the 17 target genes and were used to assess expression levels with qRT-PCR (Table 2). Of the
252 sequences tested for suitability for expression normalisation, PUBQ and RIB were the most
253 stable with *M* (gene stability) values of 1.102 and coefficients of variation (CV) values of
254 0.414 and 0.412, respectively (Hellemans et al. 2007). The *M* value for PP2A was 1.411 and
255 thus excluded.

256

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

259 All 15 defence-related genes were differentially expressed in at least one time point following
260 inoculation of 'Genesis090' and 'Kaniva' when compared to the un-inoculated controls. Of
261 the four isolates assessed, the highly aggressive 09KAL09, produced a grossly different
262 expression profile across all 15 genes. The most down-regulation, was consistent in timing
263 and levels across both the resistant (Genesis090) and susceptible (Kaniva) genotypes (Figure
264 1). Conversely, the expression profile produced by the other highly aggressive isolate
265 (09KAN19) was not largely different in either timing or magnitude to the two less aggressive
266 isolates (09MEL04 and 09KIN11). Again, this was consistent among host genotypes (Figure
267 1, Figure 2). In general, the majority of differentially expressed genes were up-regulated as
268 early as 2 hpi and started to be down-regulated at 72 hpi (Supplementary Material 1).

269

270 ### Similarities in host gene expression trends among isolate interactions

271 Cluster analysis shown similarities in individual gene expression profiles following exposure
272 to each of the four isolates (Figure 2). Mega Cluster I contained PR2B, up-regulated as early
273 as 12 hpi in 'Kaniva' and 'Genesis090' regardless of isolate applied. Cluster II was divided
274 into six subgroups. Cluster II.1:2 comprised ERG and LZP genes, which were not up-
275 regulated when either host genotype was inoculated with either 09KAL09 or 09MEL04, but
276 were up-regulated when inoculated with 09KAN19 or 09KIN11 (as early as 2 hpi). Cluster
277 II.2:3 comprised CARNAC, GST and SDCCP. CARNAC was up-regulated in all interactions
278 except when Kaniva was inoculated with 09KAL09, suggesting that lack of expression may
279 lead to susceptibility when inoculated with the highly aggressive isolates. GST and SDCCP
280 were up-regulated in at least one time point in both genotypes when exposed to any of the
281 isolates. Cluster II.3:5 comprised PAMP, RGA4, RGA7, TF1063 and SPK. Cluster II.4:1
282 contained TF1082, Cluster II.5:2 and Cluster II.6:1 comprised RGA10&5, and SN2,
283 respectively. Genes in Cluster II 3, 4, 5 and 6 were up-regulated in both genotypes infected
284 with 09KAN19 and 09KIN11 as early as 6 hpi. When infected with 09MEL04, these genes
285 were up-regulated at later infection stages (>12 hpi), but remained at either a basal expression
286 rate or were down-regulated when the genotypes were infected with 09KAL09.

287

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

290 Following inoculation with isolate 09KAL09, major differences in the gene differential
291 expression profiles were observed among the ten host genotypes assessed, which ranged in
292 classification from resistant to susceptible (Supplementary material 2; Figure 3). The mean
293 expression profiles of each genotype at a 95 % confidence interval identified six genes,
294 CARNAC, ERG, GST, RGA4, SN2 and TF1082, differentially expressed across all ten host

295 genotypes (Supplementary material 2). The expression of PR2B was not differential but
296 consistently highly up-regulated among all hosts. PAMP was up-regulated in all hosts except
297 for the highly susceptible Kaniva, perhaps indicating a lack of recognition. To further identify
298 which genes were up-regulated in the resistant and moderately resistant genotypes in
299 comparison to the susceptible genotypes, the mean expression profiles of each genotype were
300 categorised into their susceptibility levels and compared (Supplementary material 3 and
301 Supplementary material 4). Between moderately resistant and resistant genotypes, only SN2
302 was differentially expressed, more highly in moderately resistant genotypes. Interestingly, no
303 genes were differentially expressed between moderately resistant and moderately susceptible
304 classified genotypes at any of the time points assessed, however, four genes, SN2, GST, ERG
305 and RGA4, were differentially expressed between resistant/moderately resistant and
306 susceptible genotypes. Three, SN2, GST, and ERG, were expressed at higher levels in
307 resistant/moderately resistant than susceptible genotypes and one, RGA4, at a higher level in
308 susceptible genotypes. Following validation across a broader germplasm and in response to a
309 larger number of isolates, the differential expression of these four genes may be useful as
310 tools for future molecular selection of resistance within breeding programs.

311

312 **Discussion**

313 For the first time, they study has demonstrated that *A. rabiei* isolates of a similar high
314 aggressiveness level are able to cause different host responses within the same chickpea
315 genotype. One might postulate that 09KAL09 is able to evade detection and recognition and
316 then goes on to suppress host defence responses whilst it establishes itself and begins to
317 evade and colonise the tissues. Meanwhile 09KAN19, also highly aggressive, is detected and
318 recognised almost immediately (and certainly by 2 hpi), causing the up-regulation of the

319 spectrum of defence responses related to the genes under study. The question remains
320 regarding what differentiates the ability for the 09KAL09 isolate to be highly aggressive
321 compared to the two less aggressive isolates. Perhaps other isolate-related fitness
322 characteristics are important in establishing and maintaining infection ahead of host
323 defences? Certainly, the timing of gene expressions was largely indifferent following
324 exposure to the highly aggressive 09KAN19 or either of the less aggressive isolates,
325 indicating that molecular evidence of pathogenicity differences among these three isolates
326 was not captured in this study and on these cultivars, hence a wider range of defence-related
327 genes and cultivars would need to be assessed. This would be more feasible with whole
328 genome transcriptomics in response to *A. rabiei* inoculation.

329 Another plausible reason to the down regulation of most differentially expressed genes in
330 both susceptible and resistant genotypes is the production, deletion or selection of fungal
331 effectors in 09KAL09 that impact on pathogen recognition. This may trigger different host
332 defence mechanisms. Positive selection occurring within the effector proteins has been
333 observed quite extensively for *Phytophtora sojae* of soybean (Jiang et al. 2008). This was
334 postulated as a mechanism employed to enable escape from host resistance protein detection
335 and potentially adapt to different host virulence targets (Ellis et al. 2009). Indeed, mutation of
336 motifs in the C-terminus of an Avr1b protein reduced the ability of the pathogen to suppress
337 programmed cell death (PCD) and also abolished the avirulence interaction of Avr1b with the
338 *Rps1b* resistance gene in soybean (Dou et al. 2008). A similar mechanism may be occurring
339 within *A. rabiei* isolate 09KAL09, enabling it to evade detection by chickpea.

340 During industry establishment, selection of a narrow gene pool and subsequent inbreeding
341 has led to a lack of genome diversity across cultivated chickpea, which has also likely
342 constricted the potential diversity of defence mechanisms retained within Australian chickpea
343 cultivars. This low diversity of defence mechanisms was shown in the considerably fewer

344 number of disease resistance gene homologues in chickpea in comparison to other legume
345 species (Varshney et al. 2013). However, significant differences in expression levels and
346 timings of the 15 defence-related genes assessed in the current study were detected among the
347 10 host genotypes assessed. At very early time points (2-6 hpi) these are likely related to
348 differences in the timing of pathogen recognition and subsequent speed to signal down-
349 stream defence mechanisms.

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

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

367 Meanwhile, *CaETR1* (*Cicer arietinum* L. Ethylene receptor-like sequences) was the first
368 ethylene receptor discovered in chickpea associated with *A. rabiei* resistance (Madrid et al
369 2010). The ERG locus is closely linked to a major QTL, QTL_{AR1} proposed to condition
370 resistance to pathotype II (Iruela et al. 2006; Madrid et al. 2012). Recently, the *CaETR1* and
371 *CaETR-1a*/*CaETR-1b* alleles from resistant and susceptible chickpea genotypes (Madrid et
372 al. 2012) were used to negatively select and eliminate susceptible individuals from a breeding
373 program (Madrid et al. 2013). The differential expression of the allele (unknown) observed in
374 the current study between resistant/moderately resistant and susceptible genotypes may
375 further indicate its suitability for resistance selection across broad range of germplasm.

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

389 Meanwhile, PR proteins are pathogen-induced proteins classified into 17 families from PR-1
390 to PR-17, based on biochemical properties (Van Loon et al. 2006). As observed for PR2B in
391 the current study, they may be expressed prior to infection due to involvement in plant

392 development (Edereva 2005), accumulated and synthesized for a long lag period. They may
393 then be translocated from the site of induction to other plant parts during pathogenic attack
394 (Matsuoka and Ohashi 1986). Ultimately, PR2B (β1, 3-glucanase) produces glycosidic
395 fragments which weakens and decomposes fungal cell walls containing glucans, chitin and
396 proteins (Kombrink and Schmelzer 2001; Edereva 2005). As previously reported, the PR2B
397 gene was significantly up-regulated at 48 to 72 hpi compared to other time points,
398 particularly in 'Genesis114', 'PBA HatTrick', 'Almaz', 'Genesis090' and 'Kaniva' (Hanselle
399 and Barz 2001; Coram and Pang 2006; Cho and Muehlbauer 2004).

400 The gene deemed to regulate polymorphic antigen proteins (PAMP) was up-regulated at 12
401 and 48 hpi in all genotypes except for the susceptible 'Kaniva'. This gene is likely to be a
402 homologue of Enolase phosphatise E1 protein, a bifunctional enzyme of methionine salvage
403 that regenerates methionine from 5'-methylthioadenosine (MTA) (Wang et al. 2005). Its
404 function in the resistance response is likely via its metabolism that utilises polyamines (PAs),
405 nicotianamines (NAs) and interacts with ethylene biosysnthesis (Waduwara-Jayabahu et al.
406 2012). PAs are associated with cell division as a response to abiotic and biotic stress
407 (Takahashi and Kakehi 2010; Vera-Sirera et al. 2010). NAs act as chelators for long distance
408 ion transport and defence signalling processes (Curie et al. 2009), and ethylene is a
409 phytohormone capable of signalling within defence pathways.

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

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

428

429 **Author contributions**

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

434

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437

438 **Conflict of interest**

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

440

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

Genotype	Disease rating	Citation
Genesis090	Resistant (R)	Pulse Australia (2009c)
PBA HatTrick	Resistant (R)	Pulse Breeding Australia (2009)
90102-5Q-1103	Resistant (R)	K. Hobson (pers. comm.)
94-121*99V4006	Resistant (R)	K. 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)

658

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

Gene ID	Gene name (abbreviation)	Biological significance	Primers	Primer conc (µM)	Product size (bp)	Reference
CV793598	Pathogenesis-related protein 2B (PR2B)	β-1,3-Glucanase – hydrolysis of flavonoid and isoflavonoid compounds	F: GCCTAGAAAGGCAAATCCTTC R: CATCTGCCGTGGGAATAAGA	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	205	
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	
CR955005	Resistance gene analog 4 - LG8 (RGA 4)	Chickpea RGA families linked to NBS-LRR genes	F: GGCCATTGAATCAAGACGAG R: CACATTTCACCACAATCTCC	0.30	113	Palomino et al. 2009
DY396288	Resistance gene analog 5- LG2 (RGA 5)		F: GAACGACGACCAAGATAC R: CCATTTACGACTTCCGCAC	0.30	140	
AW774607	Resistance gene analog 7 - LG3 (RGA 7)		F: GCGACCGTCTTGTATGACAC R: GGAGCTTCCTGTTGTATAGCC	0.30	211	
CX533869	Resistance gene analog 10 - LG6 (RGA 10)		F: TGCCGTATTGCTGATCTGA R: TAGATGCGTTGTGAAGATT	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: TAGGGTTTGGACCAAGCAAG R: CTTCTGAGACTGCTGCAACG	0.30	151	Madrid et al. 2012
AJ515032	Polyubiquitin (PUBQ)	House keeping gene	F: AGGTGGAAAGTTCAGACAC	0.30	80	Castro et al. 2012

			AAT R: ACCTTTGCTGATCTGGTGGGA		
AJ131050.1	Ribulose 1,5-biphosphate carboxylase small subunit (RIB)	House keeping gene	F: CCACCATTGACTGAAGAGCA R: TTGAACAGCCTCAGTGCAAC	0.30	192

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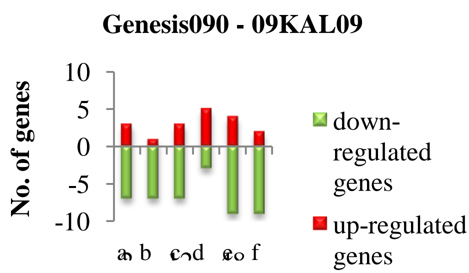

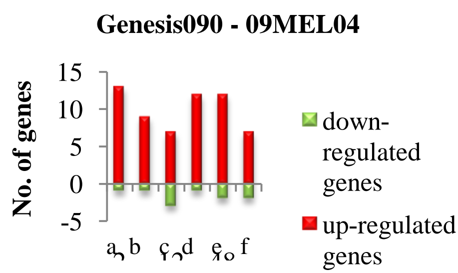

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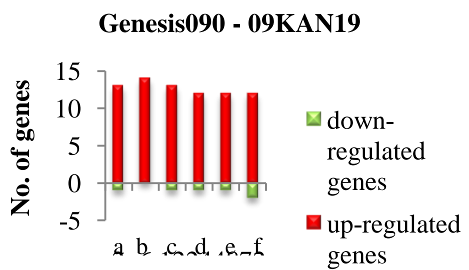

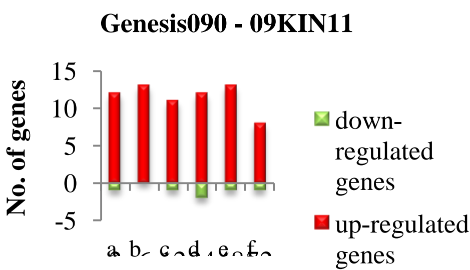

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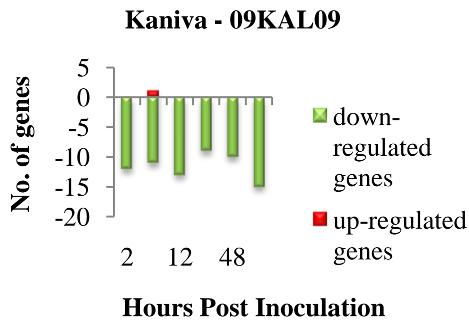

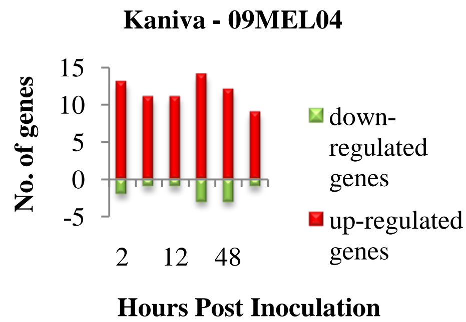

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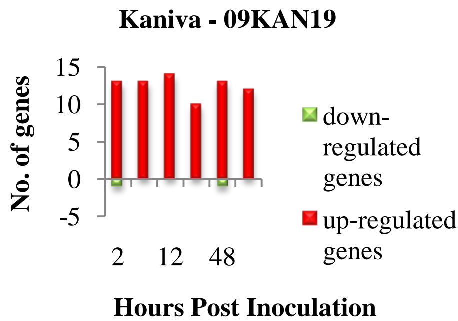

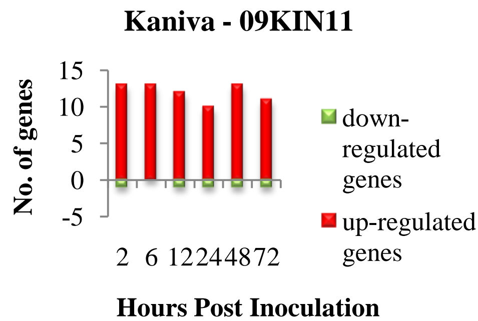

666

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

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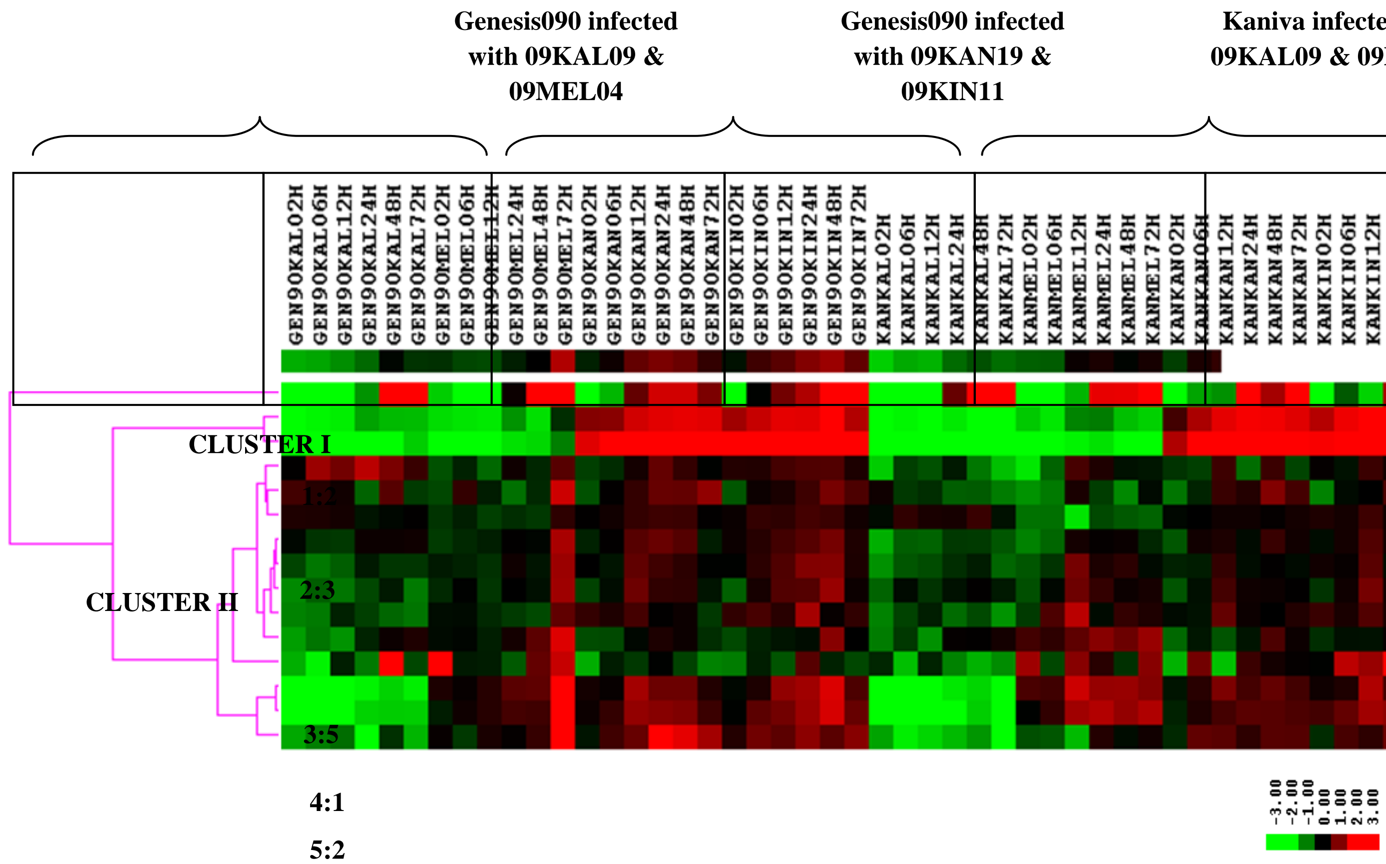
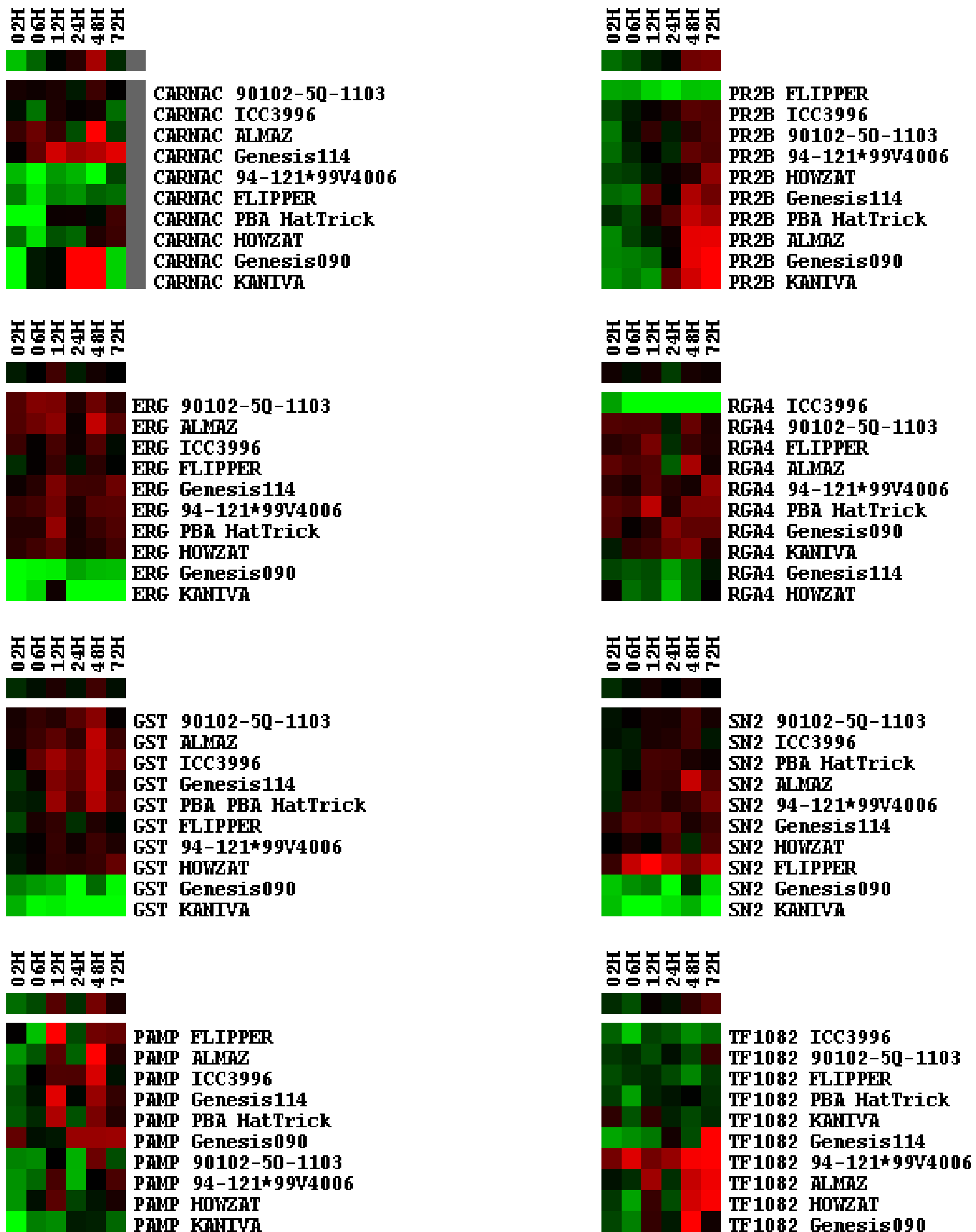


676

677 Figure 2. Heatmap of 15 genes expression profiles for Genesis090 (resistant) and Kaniva (susceptible)
678 over the time course after infection with 4 *A. rabiei* isolates, 09KAL09, 09MEL04, 09KAN19 and
679 09KIN11. Up-regulation is indicated in red, down-regulation is indicated in green, normalised
680 expression values close to the mean are in black. No detectable expression is in grey. The Log_2 values
681 of the expression profile for each treatment and genotype were normalised with two reference genes
682 and non-inoculates samples.

683



684

685 Figure 3. Heatmap of eight genes expression profiles for 10 chickpea genotypes (Table 1) over the
686 time course after infection with the most pathogenic *A. rabiei* isolate, 09KAL09. Up-regulation is
687 indicated in red, down-regulation is indicated in green, normalised expression values close to the

688 mean are in black, no detectable expression is in grey. The Log_2 values of the expression profile for
689 each treatment and genotype were normalised with two reference genes and non-inoculates samples.

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