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Transcriptome analysis reveals class IX ethylene response factors show specific up-regulation in resistant but not susceptible *Medicago truncatula* lines following infection with *Rhizoctonia solani*

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Abstract The fungal pathogen *Rhizoctonia solani* AG8 causes substantial losses to cereal and legume production in Australia and the Pacific Northwest of the United States of America. Mutant analyses have revealed a critical role for ethylene mediated defence signalling for resistance to *R. solani* AG8 in the model legume *Medicago truncatula* which is, at least in part, mediated by ethylene dependent accumulation of isoflavonoids. In this study we investigate the potential for members of the ethylene response transcription factor (ERF) family in mediating the isoflavonoid and defence response. A strong and early *Rhizoctonia*-responsive expression pattern was observed for many of the class IX *ERFs* in the moderately resistant wild type line A17, while the ethylene insensitive and highly susceptible mutant sickle (*skl*) showed a very limited regulation of this class. Conversely, the *skl* mutant demonstrated up-regulation of class II *ERFs* known to act as transcriptional repressors. Analysis of the presence of the *GCC box* promoter element, thought to be responsible for ERF binding and transcriptional activity, in genes differentially regulated in A17 suggests indirect or alternative mechanisms of *ERF* mediated gene regulation may be contributing to the large scale transcriptional adaptation of A17 following *R. solani* AG8 infection. Comparison of the expression profile with that following infection of A17 and *skl* with the symbiotic bacterium *Sinorhizobium medicae* suggests that legumes have adapted the *ERF* family to perform diverse roles to balance defence against pathogens and symbiosis with beneficial microorganisms in the same root tissue.

Keywords Necrotroph · Fungal pathogen · Root disease

The fungal pathogens belonging to the *Rhizoctonia solani* species complex cause diseases on a wide range of economically important food and fibre crops (Sneh 1991). The species complex is divided into 13 anastomosis groups (AGs) and one bridging isolate (Sneh 1991). Isolates of AG1 are responsible for Rhizoctonia foliar blight of soybean which caused substantial losses to worldwide soybean production with 14–30% losses described in Louisiana and 31–60% losses described in north and northeast Brazil (Wrather et al. 2001; Fenille et al. 2002). Rice sheath blight disease caused by isolates of *R. solani* AG1-IA cause substantial losses to rice in China, India and the USA (Savary et al. 2000; Lee and Rush 1983) and AG2-2IIIB isolates cause

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considerable losses to sugar beet in the USA and Europe (Kiewnick et al. 2001). Isolates belonging to AG8 cause bare patch disease in cereal crops in Australia and the pacific northwest of the USA and root and hypocotyl rot to legumes (Anderson et al. 2013; Okubara et al. 2014; Foley et al. 2016; Anderson et al. 2017). On wheat, *R. solani* AG8 causes characteristic spear tipping and spreading lesions on root tissue with the disruption of the root system leading to reduced growth of above ground tissues or plant death (Sweetingham and MacNish 1994).

To help address the disease issues caused by *R. solani* AG8 on legume crops, a pathosystem was recently developed for studying the interaction of *R. solani* AG8 with *Medicago truncatula* to allow the use of the extensive genetic and genomic resources, such as a genome sequence (Tang et al. 2014) and mutant populations available for the model plant. Similarly to wheat, *R. solani* AG8 infection of *M. truncatula* results in spear tipping and spreading lesions on roots, but also hypocotyl rot which together result in plant death or severe stunting (Anderson et al. 2013). Ecotype and mutant screening studies identified moderate resistance in the reference genotype A17, while the high level of susceptibility in the ethylene insensitive sickle (*skl*) mutant in the A17 background demonstrated the ethylene pathway is important for this resistance (Anderson et al. 2010; Penmetsa et al. 2008). Furthermore, resistance to AG8 could be enhanced by the addition of exogenous ethylene to A17 or by over-activating *MtERF1–1,* a class IX member of the ethylene response transcription factor (*ERF*) family in either A17 or *skl* (Anderson et al. 2010). Further analysis of the interaction identified the resistance in A17 was specifically associated with enhanced activity of the isoflavonoid biosynthesis pathway and accumulation of several isoflavonoids and related compounds (Liu et al. 2017). Despite the absence of an isoflavonoid response in the *skl* mutant, the mechanisms by which ethylene leads to the activation of the biosynthesis pathway remains unclear. The *ERF* transcription factor family, and in particular those belonging to class IX, are known to play major roles in mediating ethylene dependent defence responses (Gutterson and Reuber 2004; Nunez-Pastrana et al. 2013). The activity of the ERF transcription factor proteins is thought to predominantly be mediated through binding the *GCC box* promoter element (AGCCGCC) contained within the promoters of ethylene and jasmonate-responsive defence genes (Brown et al. 2003). This study further investigates the activity of the *ERF* family following *R. solani* AG8 infection and the potential for direct regulation of isoflavonoid biosynthesis genes.

M. truncatula seedlings of the A17 genotype or the *skl* mutant were germinated on moist filter paper in the dark for two days followed by one day at 24 °C then planted into mock-treated vermiculite or vermiculite that was pre-infested with *R. solani* AG8 (WAC10335) by placing four infected millet seeds in each 50 mm square pot in accordance with (Lichtenzveig et al. 2006). Plants were grown at 24 °C with 16/8 h light/dark photoperiod with a light intensity of 200 μmol m^{-2} s^{-1}. Root tissue was collected from pools of 16 mock-treated or infected plants at two and seven days after inoculation. The samples from three independent replicate experiments were independently subjected to RNA extraction and sequencing according to Liu et al. (2017). RNA-seq reads were trimmed using Cutadapt (Martin 2011) and mapped to the *M. truncatula* genome version 4.0 (Tang et al. 2014) using Tophat2 (Trapnell et al. 2009; Trapnell et al. 2012). The resulting BAM files were sorted using SAMtools version 0.1.19 (Li et al. 2009) and the number of aligned reads per gene counted using Htseq-count (Anders et al. 2015). The EdgeR package (Robinson et al. 2010) in R version 3.2.2 was used to normalized the data and analyse for differential expression using a FDR < 0.05 cut-off to determine differentially expressed genes. Software parameters are according to Liu et al. (2017). The resulting data is available in ArrayExpress under accession number E-MTAB-5473 and the Gene Expression Omnibus (GEO) under accession number GSE94260.

Quantitative polymerase chain reaction (QPCR) verification of RNA-seq results was conducted on an independent inoculation experiment conducted as described above and consisting of five biological replicates. Reverse transcription of RNA and QPCR was performed as previously described (Gao et al. 2007; Anderson et al. 2010) using the internal control genes *Medtr4g019110* (β-tubulin), *Medtr3g095530* (actin II) and *Medtr3g091400.1* (phosphatidylinositol 3- and 4-kinase) (Gao et al. 2007; Kakar et al. 2008; Anderson et al. 2010; Williams et al. 2016). Data were analysed by two-way ANOVA with post hoc Tukey HSD tests using the JMP 7.0 software package (SAS Institute, Cary, NC). Primers are listed in Supplementary Table 1.

Through analysis of the *M. truncatula* genome sequence, Shu et al. (2016) identified 123 putative *AP2/*

ERF genes with 20 of these classified into class IX (Shu et al. 2016). Through searching the genome sequence with the conserved *ERF* DNA binding domain, we have identified an additional two potential class IX genes (Supplementary Table 2). Of the 22 genes classified into class IX, 50% showed up-regulation in A17 following *R. solani* AG8 infection, while two of these genes, Medtr1g069960 and Medtr7g096810, also show up-regulation in *skl* at the late time point. The remaining 11 genes showed no differential expression in any sample (Table 1, Supplementary Table 2). Class IX was confirmed as the main *ERF* class to show transcription response to pathogen infection in A17 with only two other genes belonging to the *RAV* class showing up-regulation following *R. solani* infection. The expression of the Class IX *ERF* genes showing transcriptional response to *R. solani* inoculation was further assessed using QPCR on cDNAs from an independent experiment consisting of five biological replicates (Supplementary Figure 1, Supplementary Table 3). The majority of genes tested behaved similarly to the RNA-seq analysis with *Medtr1g070000*, *Medtr1g070070*, *Medtr1g074230*, *Medtr1g074370*, *Medtr7g096700* and *Medtr7g096750* showing exclusive up-regulation in A17 while the up-regulation of *Medtr1g069960* and *Medtr7g096810* following *R. solani* inoculation was confirmed in both A17 and *skl*. Despite confirmation of the expression patterns of nine out of 11 *MtERF* genes tested, two genes showed discrepancy between the RNA-seq and QPCR results. Medtr1g040430 showed a small but significant induction in A17 at 7 dpi with an average of 1.88 fold found from the three biological replicates however the QPCR identified higher variation among the five biological replicates from that experiment producing an insignificant change in expression with an average of 1.1 fold induction over the mock treated plants. By contrast the QPCR data suggest a significant induction of Medtr7g096780 in skl at 7 dpi whereas the RNA-seq data show no significant change in expression. Due to the low basal levels of expression, the read coverage for the gene Medtr7g096780 from the RNA-seq data was very low and two of the mock replicates did not pass the minimum counts per million (CPM) threshold of >1 which reduced statistical power. By contrast the QPCR was able to detect the low levels of expression of Medtr7g096780 in the skl mock and 7 dpi sample which presents as a significant induction. Both results suggest a higher sensitivity was observed in the QPCR analysis than in the RNA-seq analysis but overall the observed expression changes in response to *R. solani* were in good agreement between the two techniques.

Studies on other plant species interacting with *R. solani* pathogens have also found up-regulation of one or more *ERF* genes (Guerrero-Gonzalez et al. 2011; Foley et al. 2016), suggesting the activation of these transcriptional regulators may be a conserved mechanism of host defence against *R. solani* and further investigation of the activity of the class IX *ERFs* in these species may be warranted. Interestingly, Larrainzar et al. (2015) identified several of the class IX *ERF* genes to have an early and transient induction upon treatment of A17 with the symbiotic bacterium *Sinorhizobium medicae*. Similar to this study, these genes were not up-regulated in the *skl* mutant and the authors suggest this is consistent with transient activation of host defences by ethylene in response to the bacterium. *ERF* genes of other classes also play important roles in regulating symbiotic interactions with microbes. For example, *ERN1*, *ERN2*, *ERN3* and *EFD* (Medtr7g085810.1, Medtr6g029180.1, Medtr8g085960.1, Medtr4g008860.1) are required for normal nodulation (Cerri et al. 2016; Vernie et al. 2008; Andriankaja et al. 2007) however, none of the symbiosis related *ERF* genes showed significant changes in expression following infection with *R. solani*. Besides the early transient induction of class IX *ERF*s, there was little other similarity in the overall gene expression responses induced by *R. solani* (see GEO accession GSE94260 for a list of differentially expressed genes) or *Sinorhizobium* in A17, suggesting a tight regulation of defence responses occurs during the initiation of defence or symbiotic interactions.

Besides the up-regulation of class IX *ERFs*, there were two class II, one class III and one AP2 type *ERF* down-regulated in A17 following *R. solani* infection (Supplementary Table 2). The class II *ERF* transcription factors are known negative regulators of defence gene expression (Gutterson and Reuber 2004) and in contrast to the two class II genes down-regulated in A17, one of these was up-regulated in *skl*. The important defence suppression role played by the class II *ERFs* is demonstrated by the enhanced susceptibility of Arabidopsis lines over-expressing *ERF4* to *Fusarium oxysporum* (McGrath et al. 2005). For this reason the class II *ERFs* are thought to play a role in the tight regulation of defence responses in plants and the release from suppression by down-regulation of class II *ERFs*, such as that observed in A17, may be key to mounting an effective defence response.

Downstream defence responses activated by the class IX *ERFs* are thought be reliant on the interaction of the ERF DNA binding domain with the *GCC box* promoter element contained within the promoters of defence genes (Hao et al. 1998). To understand which potential downstream defence genes the class IX *ERFs* may be directly regulating, we examined the presence of the *GCC box* in the promoters of genes differentially regulated in A17. For each differentially regulated gene in A17, a promoter region was obtained that corresponded to 1Kb upstream of the translation start site from the *Medicago truncatula* genome Mt4.0 V1 (http://www.medicagogenome.org/downloads) and these were searched for the presence of the GCC core element AGCCGCC. Eleven instances of the element were identified in 10 of the 404 promoters analysed (Supplementary Table 4). GO term analysis of the 10 genes using GOretriever of the AGRIGO suite (McCarthy et al. 2006) and QuickGO (Binns et al. 2009) revealed that the majority had functions associated with defence responses. The defence related genes encode: pathogenesis related proteins (*Medtr7g115220.1*, chitinase; *Medtr2g435310.1*, bet VI PR protein), a TIR-NBS-LRR disease resistance like protein (*Medtr4g014140*), a flavone synthase enzyme (*Medtr4g088170.1*), and a terpene synthase (*Medtr7g057340.1*, (E)-beta-caryophyllene synthase). Although the isoflavonoid biosynthesis pathway was specifically up-regulated in A17 in response to *R. solani* in an ethylene dependent manner (Liu et al. 2017), the majority of genes contributing to this pathway do not possess the core *GCC box* in their promoters. Furthermore, the large proportion of genes up-regulated in A17 that do not contain a *GCC box* element suggests that either a substantial degree of *ERF*-independent regulation is occurring, transcriptional activation is occurring through intermediate transcription factors and/or novel promoter elements are responsible for ERF protein binding.

Despite the rarity of the *GCC box* element in the promoters of *R. solani*-responsive genes, several findings such as the large increase in susceptibility in the ethylene-insensitive *skl* mutant relative to the A17 parental line and the enhanced resistance observed when over-expressing *MtERFs*, point to an important role for the class IX *ERFs* in defence against *R. solani* in legumes. The high degree of specificity of up-regulation of the class IX subgroup of the large *ERF* family suggests this resistance may be regulated to a considerable degree by members of this class which may also be involved in controlling early stage infections by symbiotic microorganisms. Furthermore, the absence of *R. solani*-responsive expression of the early nodulation associated *ERFs*, *ERN1*, *ERN2*, *ERN3* and *EFD* suggest that legumes have adapted the *ERF* family to perform diverse roles to balance defence against pathogens and symbiosis with beneficial microorganisms in the same root tissue.

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Compliance with ethical standards

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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

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