VelA and LaeA are key regulators of the *Epichloë festucae* transcriptomic response during symbiosis with perennial ryegrass

Authors

M. Rahnama*[a,b,d], P. Maclean[a], D.J. Fleetwood[a,c], and R.D. Johnson*[a]

[a]AgResearch, Grasslands Research Centre, Palmerston North, New Zealand

[b]School of Biological Sciences, University of Auckland, New Zealand

[c]Biotelliga Ltd, Auckland, New Zealand

[d]Current position: Department of Plant Pathology, University of Kentucky, USA

*Corresponding author: richard.johnson@agresearch.co.nz, mostafa.rahnama@uky.edu

Abstract

VelA (or VeA) is a key global regulator in fungal secondary metabolism and development which we previously showed is required during the symbiotic interaction of *Epichloë festucae* with perennial ryegrass. In this study, comparative transcriptomics analyses of Δ*velA* mutant compared to wild type *E. festucae,* under three different conditions (in culture, infected seedlings and infected mature plants) were performed to investigate the impact VelA on the *E. festucae* transcriptome. These comparative transcriptomics studies showed that VelA regulates the expression of genes encoding proteins involved in membrane transport, fungal cell wall biosynthesis, host cell wall degradation and secondary metabolism, along with a number of small secreted proteins and a large number of proteins with no predictable functions. In addition, these results were compared with previous transcriptomics experiments studying the impact of LaeA, another key global regulator of secondary metabolism and development that we have shown is important for the *E. festucae*- perennial ryegrass interaction. The results showed that although VelA and LaeA regulate a sub-set of *E. festucae* genes in a similar manner, they also regulated many other genes independently of each other suggesting specialised roles.

Background

Epichloë endophytes are symbiotic fungi that systematically colonize intracellular spaces of cool-season grasses of the sub-family Pooideae [1-3]. Most of these symbiotic interactions are mutually beneficial with the plant providing the endophyte with nutrients, and the endophyte protecting host plants from a range of biotic and abiotic stresses [4, 5]. The bioprotective effects of *Epichloë* endophytes are mediated by *in planta* production of four different well characterised classes of alkaloids: indole-diterpenes, ergot alkaloids, lolines and peramine [6].

During this mutualistic interaction, the fungus colonises most of the upper ground parts of the plant in a tightly regulated and synchronized manner [7]. Hyphae only grow in the intercellular spaces, from the meristem to the inflorescence, except for vascular bundles [2, 7]. Also, fungal hyphae continue to grow during host cell growth but cease growth when the host stops growing, although hyphae remain

55 metabolically active [8]. This results in a seldom-branched intercellular network of
56 hyphae, parallel to the leaf axis with hyphae tightly linked with the walls of
57 neighbouring plant cells [7]. A recent study has suggested that this hyphal network
58 is a result of coordinated hyphal growth and death [9].

59 Although the molecular mechanisms that regulate the mutualistic interaction
60 between *E. festucae* and perennial ryegrass is still largely unknown a number of
61 studies have shown the importance of genes required for hyphal anastomosis (soft
62 gene [10]), fungal biology and development (*velA* gene [11] and *laeA* gene [12]),
63 localised production of reactive oxygen species (required to maintain hyphal
64 polarity [13-16]) and iron homeostasis (*sidN* gene [17, 18]). Although it is not clear
65 how *Epichloë* suppresses or avoids plant responses to establish and maintain a
66 compatible interaction, recent studies have suggested that reducing the chitin
67 inducible host defence response, by altering fungal cell wall chitin, is a possible
68 mechanism [19, 20].

69 Transcriptomics has also been used to investigate the *Epichloë* interaction
70 with grasses. These studies mostly focused on comparing endophyte free grasses
71 with infected grasses [21-23]. In 2010, the interaction of *Epichloë festucae*/*Lolium*
72 *perenne* was investigated by comparative transcriptomics of wild type infected
73 plants versus a mutant deleted in the stress-activated MAP kinase gene, Δ*sakA*, -
74 infected plants by Illumina mRNA sequencing. In this study, around 11% of *E.*
75 *festucae* genes were differentially expressed, with around 75% of these genes up-
76 regulated in Δ*sakA*-infected plants compared with wild type infected plants[24]. In
77 2015, the same group investigated the *Epichloë festucae*/*Lolium perenne* interaction
78 by using three fungal mutants that cause incompatible interactions in *L. perenne*:
79 Δ*proA,* which encodes a C6-Zn transcription factor that is essential for sexual
80 fruiting body maturation in *Sordaria macrospora*, Δ*noxA*, encoding the NADPH
81 oxidase A, and Δ*sakA*. Three comparative transcriptomics analyses of infected
82 perennial ryegrasse with each of these mutants compared to wild type *E. festucae*
83 infected plants was performed by RNA-seq. 182 genes were found that were
84 differentially expressed in all three comparisons and these were proposed as the core
85 fungal gene set distinguishing mutualistic from antagonistic symbiotic states [20].

86 The velvet gene, *veA* or *velA,* is a member of the velvet family of genes that
87 normally include four members, *velA*, *velB*, *velC* and *vosA*. VelA is an important
88 conserved fungal regulator of a variety of growth and developmental characteristics
89 [25]. In *E. festucae*, we showed that *velA* is required for fungal biology and
90 development and establishing and maintaining the mutualistic interaction of the
91 fungus with its host perennial ryegrass [11].

92 Although, based on a crystal structure and yeast one-hybrid experiments
93 with the VosA protein, it appears that the velvet domain mediates DNA binding the
94 biochemical mechanism through which VelA exerts control over gene expression is
95 not fully described [26]. Comparative transcriptomics has been used to elucidate the
96 regulatory effects of different genes on fungal transcriptome profiles. Studies of
97 Δ*velA* differential expression compared with wild type fungi in different conditions
98 have mostly used microarrays. These studies showed that VelA regulates genes
99 involved in different fungal developmental and metabolism processes, coordinating
100 with functional analysis of the mutants. These processes include secondary
101 metabolism and morphogenesis in *Penicillium chrysogenum* [27], aflatoxin
102 biosynthetic genes in *Aspergillus flavus* [28] and fungal development and secondary
103 metabolism in *Fusarium fujikuroi* [29]. In *A. fumigatus* and *Aspergillus nidulans*
104 VelA is a global regulator of secondary metabolism [30, 31]. In *A. flavus* it was
105 shown that VelA regulates a broad range of genes, especially secondary metabolism,
106 with 28 of 56 predicted secondary metabolite gene clusters differentially expressed
107 [32]. In *Fusarium graminearum* VelA is involved in regulating various cellular
108 processes [33]. In *Botrytis cinerea* a comparative transcriptomics analysis revealed
109 VelA regulatory effects on fungal genes in a pathogenic interaction with its host
110 plant *Phaseolus vulgaris* including transporters, glycoside hydrolases and proteases
111 [34]. A further study of this fungus growing on solid medium showed that VelA was
112 involved in the regulation of genes encoding SM-related enzymes, carbohydrate-
113 active enzymes, and proteases [35].

114 LaeA is a global fungal regulator and a predicted interaction partner of VelA
115 [36] which we recently reported is required for *E. festucae* metabolism and
116 development and for establishing and maintaining a successful symbiotic interaction
117 with perennial ryegrass [12]. In addition, a comparative transcriptomics analyses
118 during the early stages of interaction of inoculated perennial ryegrass seedlings with

119 the Δ*laeA* mutant and wild type *E. festucae* suggested a regulatory role for LaeA on
120 the expression of genes for plant cell wall degradation, fungal cell wall composition,
121 secondary metabolism and small-secreted proteins [12, 37].

122 Based on the knowledge of regulatory roles of VelA on different fungal
123 transcriptome profiles and our findings of VelA importance in *E. festucae*
124 metabolism and development and successful symbiosis [11], we hypothesised that
125 VelA may be involved in regulating the *E. festucae* transcriptome. In this study, a
126 set of comparative transcriptomics analyses of Δ*velA* mutants versus wild type *E.*
127 *festucae* in culture, in infected seedlings and in infected mature plants was
128 performed. In addition, these results were also compared to the transcriptomics
129 analysis of Δ*laeA* versus wild type *E. festucae* in seedlings [12, 37] and in culture.
130 We identified a specific set of VelA regulated genes that define possible processes
131 required for this symbiotic interaction.

132 Results

133 Choosing the conditions for transcriptomics studies

134 The regulatory effects of VelA on *E. festucae* were determined using
135 comparative transcriptomics analysis of wild type and Δ*velA* mutant strains grown
136 under three different conditions; in culture, during the early stage of infection (in
137 seedlings) and in mature infected plants (Table 1). These results were also compared
138 to the transcriptomics analysis of Δ*laeA* versus wild type *E. festucae* in seedlings
139 [12, 37] and in culture.

140 In order to choose the best time for the transcriptomics study, *velA*
141 expression was examined in wild type *E. festucae* under the three different
142 conditions (in culture, in seedlings and in mature infected plants) employed.

143 As we showed previously, the highest *velA* expression in culture is under
144 nutrient deprivation (water agar plates) and the lowest expression is in nutrient
145 replete medium (PDA) [11]. Similar results were observed for *laeA* expression in
146 different media [12]. Because for artificially infecting ryegrass plants with *Epichloë*,
147 normally fungi that is growing for two weeks in PDA medium use as inoculum, for
148 in culture transcriptomics study also same fungi in PDA medium was used.

149 For the seedling study, expression of the *velA* gene was examined after
150 inoculating seedlings with wild type *E. festucae*, growing in both the light and dark,
151 at four time points post-inoculation (24 HPI, 2.5 DPI, 6 DPI and 14 DPI). Expression
152 of *velA* increased over time and showed the highest expression 14 DPI. At all-time
153 points, higher expression was detected from seedlings growing under light compared
154 to the dark (Fig1a). As we showed before, the highest *laeA* expression was also at
155 14 DPI although it was not a gradual increase as was the case with *velA* [12]. In
156 addition to *velA* and *laeA* having the highest expression at 14 DPI, this time point
157 also correlated with when Δ*velA* and Δ*laeA* mutant inoculated seedlings began to die
158 [11, 12]. Because of these reasons, 14 DPI was chosen to study the requirement of
159 *velA* and *laeA* on the expression profile of *E. festucae* during infection of perennial
160 ryegrass.

161 In mature plants, as we showed before, old blades have the highest *velA*
162 expression compared to other tissues [11] so this was also used in the current
163 transcriptomics study.

164 The relative expression of *velA* and *laeA* in culture, in seedlings and in
165 mature plants was assessed using qRT-PCR. *velA* showed higher expression in
166 seedlings compared to mature plants. This is opposite to the expression of *laeA* under
167 the same conditions suggesting that *velA* and *laeA* exert different effects on the early
168 stage of infection (Fig. 1b).

169 **General description of RNA-sequencing results**

170 Higher numbers of reads were generated from in culture samples compared
171 to seedlings and mature plants (Fig. S1a and Table S2). Although similar numbers
172 of reads were generated for both seedling and mature plant samples (Fig S1a) only
173 1.84% of total reads from mature plants mapped to the *E. festucae* genome compared
174 to 6.28% from seedlings (Table S2) which is indicative of the higher fungal biomass
175 (relative to plant tissue) in seedlings.

176 Multidimensional scaling (MDS) analysis of the 1,000 most highly
177 expressed genes shows that all in planta replicate samples clustered very close to
178 each other but were separated from in culture samples (Fig. S1b).

179 Genes with equal or greater than two times fold changes and a FDR (false
180 discovery rate) equal or less than 0.05 were counted as differentially expressed genes
181 (DEGs) (Fig 2a). The percentages of DEGs observed in the S Δ*velA*-WT comparison
182 was higher than other *velA* and *laeA* related comparisons. In S Δ*velA*-WT
183 comparison 5.1 and 1.3 times more DEGs compared to IC Δ*velA*-WT and IP Δ*velA*-
184 WT comparisons were observed which indicates a significant impact of the growth
185 conditions on the number of DEGs in the Δ*velA* mutant. Only 10% and 23.5% of
186 DEGs (29 and 69 genes) in the S Δ*velA*-WT comparison was common with DEGs
187 in the IC Δ*velA*-WT and IP Δ*velA*-WT comparisons, respectively (Fig 2b).

188 In the IC Δ*laeA*-WT comparison there was almost 2 times more DEGs
189 compared to IC Δ*velA*-WT and only 15.38% (22 genes) of them are common (Fig.
190 2a and b). DEGs in S Δ*laeA*-WT are around 26% less than S Δ*velA*-WT but similar
191 to IP Δ*velA*-WT (Fig. 2a). More than 69% of the DEGs in S Δ*laeA*-WT are
192 downregulated but in both S Δ*velA*-WT and IP Δ*velA*-WT a similar percentage of
193 DEGs are up and down-regulated (Fig. 2a). DEGs in S Δ*laeA*-WT comparison had
194 52% and 31% (112 and 67) in common with S Δ*velA*-WT and IP Δ*velA*-WT
195 comparisons, respectively, which indicates different regulatory effects of LaeA and
196 VelA on the fungal transcriptome during interaction with its host (Fig. 2b).

197 There are 182 DEGs common in transcriptomes of three *E. festucae* mutants
198 (Δ*proA*, Δ*noxA*, and Δ*sakA*) which were proposed to form a core set of *Epichloë*
199 genes that distinguished mutualistic from antagonistic symbiotic states [20]. Our
200 results for the S Δ*velA*-WT and IP Δ*velA*-WT comparisons showed that only 46 and
201 49 genes were in common with the proposed core set from Eaton *et al*, respectively
202 (Fig. 2b). Thus, not all the core genes identified in the Eaton *et al.* (2015) study are
203 required for distinguishing mutualistic from antagonistic symbiotic states.

204 IP Δ*velA*-WT fold change range (524.7 to -99.8) was 13 and 7 times greater
205 compared to IC Δ*velA*-WT (22.3 to -11.4) and S Δ*velA*-WT (13.2 to -66.2)
206 comparisons, respectively (Fig. 2c and Fig. S1c). Interestingly, IC Δ*laeA*-WT (33.7
207 to -16.0) and S Δ*laeA*-WT (12.6 to -56.3) showed similar fold change ranges with
208 IC Δ*velA*-WT and S Δ*velA*-WT, respectively (Fig. 2c and Fig. S1c).

209 **Gene Ontology (GO) enrichment analysis on DEGs**

210 DEGs were classified based on their primary functions into 'Molecular
211 Function' and 'Biological Process' GeneOntology (GO) categories (Fig. S2 and
212 Table S3). 55-72% of DEGs in different comparisons were not aligned with any GO
213 category so it was decided that GO was not a good method to study the functions of
214 DEGs in this study.

215 **Functional annotations of differentially expressed *E. festucae* genes**

216 To further investigate the DEGs functions, BLAST analyses were performed
217 against different databases including Uniprot, Swissprot, InterProScan and KEGG
218 and their putative functions were manually determined. In 36.8% (21), 46.1% (135),
219 and 46.2% (104) of DEGs in IC Δ*velA*-WT, S Δ*velA*-WT, and IP Δ*velA*-WT
220 comparisons, respectively, no significant BLAST hits (p-value ≤ E-20) were found.
221 This suggests that many of the genes regulated by VelA are likely to be unique to *E.*
222 *festucae*. Similar numbers of DEGs genes were found with no significant hits in IC
223 Δ*laeA*-WT and S Δ*laeA*-WT with 35.2% (38) and 22.2% (48), respectively.

224 **Changes in expression of genes encoding orthologues of velvet family**
225 **members**

226 We previously reported orthologues of velvet family members in *E.*
227 *festucae*: VelA (EfM3.049680), VelB (EfM3.023360), VelC (EfM3.009960), VosA
228 or VelD (EfM3.010530) and LaeA (EfM3.069170) [11, 12]. Differential expression
229 of these genes in different comparisons showed that *laeA* expression in IC Δ*velA*-
230 WT and S Δ*velA*-WT comparisons was up regulated. However, *velA* expression did
231 not change significantly in Δ*laeA* mutant related comparisons (Table S4) which
232 suggests that whilst VelA negatively regulates the expression of the *laeA* gene, LaeA
233 does not have any significant influence on the expression of *velA*. The expression of
234 other members of the velvet family did not changed significantly in any of the Δ*velA*
235 or Δ*laeA* mutants (Table S4).

236 **DEGs in different functional categories**

237 Further analysis focussed on five areas of fungal cellular function and

238 metabolism: fungal cell membrane transporters, fungal cell wall biosynthesis, host
239 cell wall degradation, secondary metabolism and small-secreted proteins. These
240 five areas include most of the DEGs that were previously reported as important in
241 the *E. festucae*/*L. perenne* interaction [20].

242 **1. Changes in expression of genes encoding membrane transporters**

243 DEGs that encode transporters are summarised in table 2. For the in culture
244 comparison, there were a small number of DEGs (4) with transporter activity but it
245 significantly increased (11-15) in S Δ*velA*-WT, S Δ*laeA*-WT and IP Δ*velA*-WT
246 comparisons. This indicates a greater regulatory effect of VelA and LaeA on the
247 expression of *E. festucae* genes encoding membrane transporters during fungal
248 interaction with ryegrass. Higher numbers of DEGs with transporter activity was
249 observed in both *velA* related comparisons (13 and 15 for S Δ*velA*-WT and IP Δ*velA*-
250 WT comparisons) with greater fold changes (2.1-12.5) than S Δ*laeA*-WT
251 comparison (11 DEGs with 2 - 4.8 fold change range). This suggests stronger
252 regulatory effects of VelA compared to LaeA on the expression of *E. festucae*
253 membrane transporters (Table 2).

254 The genes with the highest fold changes (more than four times) were
255 involved in transporting different compounds such as nitrogen, peptides, hydrogen
256 ion and carboxylic acid. There are two genes, EfM3.012390 and EfM3.039020,
257 involved in nitrogen transport. EfM3.012390, which was only differentially
258 expressed in the IP Δ*velA*-WT comparison (4.9-fold up-regulated), is a homologue
259 of a nitrate transporter, CrnA, from *Emericella nidulans* and has been shown to be
260 involved in nitrogen metabolite repression [38]. EfM3.039020, which was up-
261 regulated in both S Δ*velA*-WT and IP Δ*velA*-WT comparisons is a homologue of an
262 ammonium transporter, Amt1, from *Schizosaccharomyces pombe* [39].
263 EfM3.027570 was up-regulated in both S Δ*velA*-WT and IP Δ*velA*-WT comparisons
264 and is a homologue of a peptide transporter, PTR2, from *Stagonospora nodorum*
265 [40].

266 These results suggest that Δ*velA* and Δ*laeA* mutants may be nutrient starved
267 during interaction with seedlings or mature plants.

268 **2. Changes in expression of genes encoding enzymes with host cell wall**
269 **degrading activity**

270 Fundamentally, plant cell walls are made of embedded cellulose microfibrils
271 in a matrix of pectin, hemicellulose and cell wall associated proteins [41]. To look
272 specifically at the enzymes with plant cell wall degrading characteristics, the Fungal
273 PCWDE (Plant Cell Wall-degrading Enzymes) Database [42], was utilised. In this
274 database there are 22 known fungal gene families which degrade the plant cell wall
275 [42]. Searching against this database showed that 25 genes in 13 PCWDE families
276 are present in *E. festucae* (Table S5).

277 In addition, the larger Carbohydrate-Active enZYmes (CAZyme) database
278 [43], which includes the families of enzymes that assemble, modify, or breakdown
279 oligo- and polysaccharides was checked [43]. The presence of 310 genes in *E.*
280 *festucae* that are homologues with CAZyme families [20] were analysed for DEGs
281 across the different comparisons (Fig. S3). The number of DEGs with homologues
282 to CAZyme families was almost ten times and two times increased in S Δ*velA*-WT
283 compared to IP Δ*velA*-WT and IC Δ*velA*-WT, respectively, and two times increased
284 in S Δ*laeA*-WT compared to IC Δ*laeA*-WT (Fig. S3). These results indicate the
285 importance of VelA and LaeA on the expression of CAZyme genes during the *E.*
286 *festucae* interaction with its host plant.

287 All DEGs with plant cell wall degrading activity are summarised in table 3.
288 No DEGs belonging to cellulases, xylanases, cutinases or pectinases were detected
289 for the in-culture comparisons. No DEGs with cellulose degradation activity was
290 detected for S Δ*velA*-WT and IP Δ*velA*-WT comparisons but two DEGs with
291 cellulose activity were detected in the S Δ*laeA*-WT.

292 Three DEGs, EfM3.040190, EfM3.005420 and EfM3.037040, with
293 hemicellulose activity were detected (Table 3). Two of them, EfM3.037040 and
294 EfM3.040190, were up-regulated in all S Δ*laeA*-WT, S Δ*velA*-WT and IP Δ*velA*-WT
295 comparisons, with the IP Δ*velA*-WT comparison showing the highest fold change of
296 all DEGs with plant cell wall degradation activity (Table 3). EfM3.037040 is a
297 homologue of xylanase C (XynC) from *Cellvibrio japonicus* which hydrolyses
298 hemicellulose (Kellett et al., 1990). This gene was 4.3, 4.3 and 69.6 fold up-regulated
299 in Δ*laeA*-WT, S Δ*velA*-WT and IP Δ*velA*-WT comparisons, respectively.

300 EfM3.040190 is a homologue of endo-1,4-beta-xylanase 2 (*xyl2*) from *Claviceps*
301 *purpurea* [44] and was 3.3, 3.9 and 83.5 fold up-regulated in S Δ*laeA*-WT, S Δ*velA*-
302 WT and IP Δ*velA*-WT comparisons, respectively. In addition to these two genes
303 another DEG was observed with hemicellulose activity, EfM3.005420, that is a
304 homologue of exo-1,4-beta-xylosidase (*bxlB*) from *Aspergillus flavus* and which was
305 2.3-fold up-regulated in S Δ*velA*-WT.

306 One DEG, EfM3.008730, 2-fold was up-regulated in the S Δ*velA*-WT
307 comparison and encodes a pectinase with homology to pectin methyl esterase (*pme1*)
308 in *Aspergillus aculeatus*. It has been shown that Pme1 in *A. aculeatus* degrades the
309 host plants cell wall [45].

310 A down-regulated DEG, EfM3.005300, identified from both seedlings and
311 in planta comparison which is a homologue of the cuticle degrading protease from
312 the insect pathogen *Metarhizium anisopliae* [46].

313 Based on these data it appears VelA regulates *E. festucae* genes that encode
314 proteins with a range of plant cell wall degrading functions during the fungal
315 interaction with its host. Of the surviving plants infected with the Δ*velA* mutant, there
316 was significantly greater hemicellulose degradation activity which may contribute
317 to the observed phenotypes of mutant infected plants [11].

318 **3. Changes in expression of genes encoding proteins involved in fungal**
319 **cell wall composition**

320 Chitin, glycoproteins and glucan are the main components of the fungal cell
321 wall and these can function as elicitors of plant defence responses [47, 48]. Fungi
322 use a range of enzymes to break down, synthesise or remodel their cell wall [49]. All
323 detected DEGs that encode proteins related to the fungal cell wall are summarised
324 in table 4.

325 Enzymes for the synthesis and breakdown of chitin are chitin synthases and
326 chitinases, respectively [49]. EfM3.000810 is a homologue of a chitinase
327 (BDCG_06828) from *Blastomyces dermatitidis* and was 3, 3.3 and 3.1 fold down-
328 regulated in S Δ*laeA*-WT, S Δ*velA*-WT and IP Δ*velA*-WT comparisons, respectively.
329 EfM3.024310 is a homologue of Endochitinase B (*chiB1*) from *Neosartorya*

330 *fumigata* [50] and was 2.1-fold up-regulated in the IP Δ*velA*-WT comparison.
331 EfM3.049120 is a homologue of chitin synthase 1 (*chs-1*) from *Neurospora crassa*
332 and was 2-fold up-regulated in the IC Δ*velA*-WT comparison. This gene has been
333 shown to play a major role in cell wall biogenesis in this fungus [51].

334 No DEGs that encode β-1,3-glucan synthase enzymes, responsible for
335 synthesis of fungal cell wall glucan, were found in *E. festucae* but there were two
336 genes, EfM3.056450 and EfM3.056810 that are possibly engaged in breaking down
337 glucan. EfM3.056450 is a homologue of EPD1 from the dimorphic yeast *Candida*
338 *maltosa*. In this fungus, it was shown that EPD1 is necessary for fungal transition to
339 the pseudohyphal growth form. Deleting this gene leads to a reduction of both alkali-
340 soluble and alkali-insoluble β-glucan levels [52]. EfM3.056450 also has a
341 glucanosyl transferase domain that in some cases has been shown to remodel chains
342 of β -1,3-glucan in the fungal cell wall [53]. This gene is up-regulated 2.1-fold in the
343 IP Δ*velA*-WT comparison. EfM3.056810 is a homologue of glucan endo-1,3-beta-
344 glucosidase from *Cellulosimicrobium cellulans* and was 2.1-fold down-regulated in
345 the IP Δ*velA*-WT comparison. This enzyme was shown to be involved in breaking
346 down fungal cell walls by hydrolysis of cell wall β-1,3-glucosidic linkages [54].

347 Glycoproteins are another constituent of the fungal cell wall that are made
348 of modified proteins by *N*- and *O*-linked carbohydrates that in lots of examples are
349 glycosyl phosphatidyl inositol (GPI) anchors [49]. There are three genes,
350 EfM3.078790, EfM3.054000 and EfM3.034340, with possible activity towards
351 glycoproteins (Table 4). EfM3.078790 is a homologue of a cell wall glycoprotein
352 *sed1* from *Saccharomyces cerevisiae* that was differentially expressed in seedling
353 comparisons (2.5 fold down-regulated in S Δ*velA*-WT comparison). In *S. cerevisiae*
354 Sed1 is the most abundant cell wall- associated protein in the stationary phase and is
355 necessary for fungal resistance to lytic enzymes [55]. EfM3.054000 is a homologue
356 of a putative cell wall glycoprotein (CPUR_07530) from *Claviceps purpurea* that
357 was 2.3-fold up-regulated in S Δ*velA*-WT comparison. EfM3.034340 is a homologue
358 of the collagen α-2(IV) chain in the nematode *Ascaris suum* and was 3.4 and 3.7-
359 fold down-regulated in the S Δ*laeA*-WT and IP Δ*velA*-WT comparison. Although
360 collagen is best understood in animals it is also detected in fungal fimbriae, long (1-
361 20 μm) and narrow (7 nm) flexuous appendages on the fungal surface, which affect

362 cellular functions such as mating and pathogenesis [56]. This gene is also a
363 homologue of a putative cell wall protein (XP_001270917) in *Aspergillus clavatus*.

364 One of the fungal phenotypes observed in mature plants infected with the
365 mutant Δ*velA* was formation of intrahyphal hyphae [11]. It has been shown that
366 triggering activation of woronin bodies, important organelles that block the septal
367 pore in response to wounding, increases the formation of intrahyphal hyphae [57].
368 EfM3.049350 is a homologue of hex protein, a major woronin body protein in
369 *Neurospora crassa* which was up-regulated 2.9 times in the IP Δv*elA*-WT
370 comparison.

371 These results demonstrate that VelA is a regulator of *E. festucae* cell wall
372 composition during the fungal interaction with ryegrass.

373 **4. *velA* is required for secondary metabolite gene expression and production**

374 In order to investigate the regulatory effects of VelA on secondary
375 metabolism in *E. festucae*, differential expression of genes involved in known
376 alkaloid gene clusters (ergot alkaloids, indole-diterpenes and peramine) were
377 examined across all comparisons. In the in culture comparison, the *lpsB* gene from
378 the ergovaline pathway was up regulated (more than 9 folds) in both Δ*velA* and
379 Δ*laeA* mutants. Another gene from this pathway, *easA,* was also up regulated (2 fold)
380 in Δ*laeA* mutant (Table S6).

381 Of the 11 genes involved in ergovaline production (*EAS* cluster genes) [58]
382 only 1 gene, *lpsB*, was up-regulated in the IP Δ*velA*-WT comparisons whereas all
383 genes were down-regulated in the S Δ*velA*-WT comparisons, which was similar to
384 the S Δ*laeA*-WT comparison (Table S6, Fig 3a).

385 Of the ten known genes for indole-diterpene biosynthesis, four genes were
386 significantly down-regulated in the IP Δ*velA*-WT comparison. In the S Δ*velA*-WT
387 comparison none of the genes were differentially expressed, which is in stark
388 contrast to the S Δ*laeA*-WT comparison where eight genes were down-regulated.
389 This indicates very different regulatory roles of *velA* and *laeA* genes on fungal
390 secondary metabolite regulation in *E. festucae* (Table S6, Fig. 3a).

391 The expression of *perA,* the sole gene required for peramine biosynthesis,
392 was not differentially expressed in any comparison (Table S6, Fig. 3a).

393 The transcriptomics results were validated by qRT-PCR of *dmaW*, *ltmG* and
394 *perA*, the first gene from the ergovaline, lolitrem B, and peramine pathways,
395 respectively, in all three different conditions using wild type, mutant and
396 complemented strains (Fig. 3b). Results validated the transcriptomics results (Fig.
397 3b).

398 To correlate alkaloid pathway gene expression results, different alkaloids
399 concentrations were also measured in infected three-months-old ryegrass plants
400 infected with wild type and Δ*velA* mutant *E. festucae* (Fig. 4a). Of the seven ergot
401 alkaloid compounds that were measured two of them, lysergic acid and
402 elymoclavine, were not detected. For the remaining five compounds, the mean
403 concentrations in the wild type-infected plants were higher than Δ*velA*-mutant
404 infected plants but only ergine was statistically significant (Fig. 4a). Of the 23
405 indole-diterpene alkaloid compounds that were measured, the mean concentrations
406 of the Δ*velA*-mutant infected plants were higher with 17 of them being statistically
407 significant (Fig. 4a). For peramine the mean concentration in Δ*velA* mutant infected
408 plants was also higher but it was not statistically significant (Fig. 4a).

409 We previously reported alkaloid production in three-month-old ryegrass
410 plants infected with wild type and Δ*laeA* mutant *E. festucae* [12] (Fig. S7). The
411 differences of mean concertation of wild type infected plants to Δ*velA* mutant
412 infected plants were compared to the mean concentration differences of wild type
413 infected plants to Δ*laeA* mutant infected plants (Fig. 4b). Results show lower
414 concentrations of most of indole-diterpene alkaloids in the Δ*laeA* mutant infected
415 plants which is opposite to that seen for Δ*velA* mutant infected plants (Fig. 4b).

416 In addition to these 3 alkaloid clusters, 29 other clusters, 191 genes, which
417 putatively encoding secondary metabolites in *E. festucae* Fl1 [6] also were examined
418 (Table S6). Heat maps generated for all 32 clusters in *E. festucae* showed that most
419 of them are down regulated in Δ*velA* mutant infected plants (Fig. 5a), showing that
420 VelA positively regulates secondary metabolite gene expression in *E. festucae*. Of
421 the 32 clusters 12 contained at least one gene differentially expressed in one of the

422 Δ*velA* related comparisons, nine in IP Δ*velA*-WT, ten in S Δ*velA*-WT and three in IC
423 Δ*velA*-WT have DEGs (Fig. 5b, left panel). In the Δ*laeA* related comparisons, also
424 12 clusters contained at least one gene differentially expressed in one of the
425 comparison, eleven in S Δ*laeA*-WT and five in in IC Δ*laeA*-WT (Fig. 5b, left panel),
426 but these clusters are not all identical to the ones in the Δ*velA* related comparisons.
427 Clusters 20, 29 and 38 are unique to Δ*velA* related comparisons and clusters 10, 35
428 and 50 are unique to Δ*laeA* related comparisons (Fig. 5b).

429 Our results show that VelA, similar to LaeA, is a key regulator for secondary
430 metabolites gene expression and production in *E. festucae.* However, it seems VelA
431 and LaeA exert different regulatory effects.

432 **5. Changes in expression of genes encoding putative small-secreted proteins**

433 Microbes during infection of host plants produce effector proteins that are
434 of key interest during plant-microbe interactions. Via a range of different molecular
435 mechanisms these secreted proteins suppress or interfere with host immune
436 responses or alter the host cell physiology, generating a favourable environment for
437 infection and growth [59, 60]. Recently, 141 genes in *E. festucae* were reported as
438 putative effectors [61]. Of these, 49 were differentially expressed in at least one of
439 the comparisons described here (Fig. 6 and Table S7). Although the S Δ*vel*A-WT
440 comparison had the highest number of SSPs (Fig. 6a) the IP Δ*vel*A-WT comparison
441 had a much broader range of fold changes (72.4 and -99.8) (Fig. 6b). *velA* and *laeA*
442 showed different regulatory effects on SSPs across the different conditions (Fig. 6c)
443 with some being unique to particular conditions (Fig. 6d).

444 Most of the predicted gene products have no homologues with annotated
445 molecular function except four genes EfM3.001305, EfM3.001310, EfM3.055320
446 and EfM3.079420 (Table S7). EfM3.001305 and EfM3.001310 are homologues with
447 a killer toxin gene, *kp4*, from *Metarhizium spp* that is a lethal protein via inhibition
448 of calcium channels [62]. EfM3.055320 was only expressed in the in planta
449 comparisons and is a homologue of Cu/Zn superoxide dismutase from *Claviceps*
450 *purpurea* [6] which are involved in reducing superoxide radicals generated by host
451 plant defence mechanisms. EfM3.079420 is a homologue of a fungal hydrophobin
452 domain-containing protein in *Pochonia chlamydosporia* [63].

453 Three putative effectors, which are some of the core symbiosis genes [20]
454 showed the highest differential gene expression and were down-regulated.
455 EfM3.016770, EfM3.062700 and EfM3.014350 called *sspM*, *sspN* and *sspO*,
456 respectively, were functionally studied in Hassing et al (2019) but no clear function
457 was identified presumably due to redundancy functions of effectors. These 3 effector
458 candidates were also down regulated in all S Δ*velA*-WT, IP Δ*velA*-WT and S Δ*laeA*-
459 WT comparisons (Table S7).

460 Although only 27% (49 genes) of the Eaton et al symbiosis core genes are
461 common with DEGs in the mature infected plants with Δ*velA*, 16% (8 genes) of these
462 are SSPs, which are 57% (8/14) of the total SSP genes in the Eaton et al core set.

463 Discussion

464 The *velA* gene is a well-known regulatory gene required for different fungal
465 cellular and developmental functions, including secondary metabolism,
466 pathogenicity, sexual and asexual development and fungal morphology and growth
467 [25, 64]. In addition, we recently showed that it is required for the symbiotic
468 interaction of *E. festucae* with perennial ryegrass [11]. Here, by using comparative
469 transcriptomics, VelA regulatory effects on the *E. festucae* transcriptome profile
470 growing in different conditions (in culture, inoculated seedlings and mature infected
471 plants) were tested and the results compared with similar experiments, previously
472 reported, on the other fungal global regulator LaeA [12, 37].

473 The numbers of DEGs in S Δ*vel*A-WT and IP Δ*velA*-WT comparisons were
474 5.1 and 3.9 times, higher than IC Δ*velA*-WT, respectively. This suggests that VelA
475 has a stronger regulatory effect when growing in seedlings and mature plants under
476 likely nutrient limited conditions, compared to PDA culture, a nutrient rich
477 condition. This also correlates with our previous report that showed much higher
478 expression of *velA* under nutrient limited medium (water agar) compared to nutrient
479 rich medium (PDA) [11], which is similar to LaeA [12].

480 The small proportion of DEGs common between IC Δ*velA*-WT, S Δ*velA*-
481 WT and IP Δ*velA*-WT comparisons suggests condition dependent regulatory roles
482 of VelA on *E. festucae* gene expression, similar to LaeA. It is possible that VelA
483 forms different protein complexes in different conditions as was shown in *A.*

484 *nidulans* [25]. Other possibilities are different post-translational modifications or
485 localisation under different conditions.

486 Compared to the core symbiotic fungal gene set identified by Eaton *et al.*
487 (2015) only a small proportion of DEGs from both IP Δ*velA*-WT and S Δ*velA*-WT
488 comparisons were in common. This may partly be a result of different tissue samples
489 used for the transcriptomics analyses between the two studies. However, based on
490 the difference in hyphal in planta characteristics between mutant *velA* and *proA*,
491 *noxA* and *sakA* mutants, it is most likely VelA, like LaeA, regulates separate
492 mechanisms important in mutualistic interactions than ProA, NoxA and SakA. One
493 of the distinct differences between in planta fungal growth of *velA* and *laeA* mutants,
494 compared to *proA*, *noxA* and *sakA* mutants was chitin distribution. In *velA* and *laeA*
495 mutants, chitin distribution was very similar to the wild type interaction [11, 12],
496 whereas for *proA*, *noxA* and *sakA* mutants, chitin distribution was increased [20].

497 Comparative transcriptomics studies of Δ*velA* mutants in different fungi
498 show conserved regulatory roles of VelA on secondary metabolism, CAZyme
499 biosynthesis, morphogenesis, development and cellular metabolism [27-33, 35], that
500 are quite similar with some reported roles of LaeA [35, 65-67]. In the fungal
501 transcriptomics analysis of S Δ*velA*-WT and IP Δ*velA*-WT and S Δ*laeA*-WT
502 comparisons, up regulation was observed in the expression of genes that encode
503 putative nutrient transporters, host cell wall degrading enzymes and small secreted
504 proteins. In these three functional categories, DEGs in the IP Δ*velA*-WT comparison
505 showed higher differential expression with more genes involved, compared with
506 seedling comparisons, although total DEGs in this comparison was not higher than
507 others. This suggests that VelA has stronger regulatory influence on fungal gene
508 expression for these three functional categories during interaction with mature plants
509 compared to the early stages of infection.

510 Fungal transporters of nitrate, ammonium, peptides and carboxylic acid
511 were up-regulated in mature plants infected with the Δ*velA* mutant, while in
512 seedlings inoculated with Δ*velA* and Δ*laeA* mutants peptide and ammonium
513 transporters were up-regulated. This may suggest that in these associations, fungi are
514 under starvation conditions, especially in mature infected plants as was previously
515 suggested for *sakA*, *noxA* and *proA* mutants interactions [20]. The observed

516 abnormal in planta hyphal growth of Δ*velA* mutant fungi and invasion of vascular
517 bundles are a sign of starvation and these hyphae may be acting as a sink to absorb
518 nutrients from host phloem [11]. Our *in vitro* analyses also show that Δ*velA* and
519 Δ*laeA* mutants under starvation conditions grow abnormally but under rich
520 conditions mostly grow normally [11, 12]. Also *in vitro* analyses of *velA* expression
521 showed highest levels of their transcripts under starvation conditions [11]. Based on
522 these observations, we speculated that in *E. festucae* during in planta growth, high
523 expression of *velA* suppresses transporters and other starvation-response genes so
524 that the fungus stops growing, leading to the growth restriction observed in wild type
525 associations. Deleting *velA* in *E. festucae* changes its mutualistic interaction with
526 ryegrass to a more antagonistic one and mutant fungi caused death in most inoculated
527 plants. It has been shown that nitrogen metabolism generally plays a key role in
528 pathogenicity [68, 69]. However, other than nitrogen transporters no predicted genes
529 involved in nitrogen metabolism were found to be differentially expressed in this
530 study. Possibly, changes in nitrogen transport may contribute to the host death, with
531 increased fungal growth and insufficient nutrition for the seedling. This is supported
532 by a comparative transcriptomics study of the *Botrytis cinerea* interaction with
533 *Phaseolus vulgaris* in which it was suggested that up regulation of 2% of DEGs that
534 encode sugar, amino acid and ammonium transporters and glycoside hydrolases is
535 the reason for the virulence disability of the Δ*velA* mutant fungus [34]. It was also
536 suggested that the transcriptomics pattern in this study is a sign of the Δ*velA* mutant
537 response to nutrient starvation conditions [34].

538 In different transcriptomics analyses, VelA regulatory effects on secondary
539 metabolite gene clusters were shown in different fungi [27-31, 33, 35]. In this study,
540 we showed VelA positively regulates 48.4% of the gene clusters for secondary
541 metabolism in *E. festucae* including the two well-known clusters for ergot alkaloids
542 and indole diterpenes. The influence of VelA on ergot alkaloid gene expression in
543 mature plants was much lower than in seedlings inoculated with Δ*velA* and in fact in
544 mature plants only one gene is differentially expressed, being up-regulated compared
545 with seedlings where most genes are down-regulated. VelA regulates indole
546 diterpene gene expression only in infected mature plants, exerting a much weaker
547 influence than seedlings inoculated with the Δ*laeA* mutant. Similar to VelA, NoxA
548 and ProA were shown to have weak regulatory effects on ergot alkaloid and indole
549 diterpene gene expression in infected mature plants, although SakA has a stronger

550 positive regulatory effect [20]. The chemical concentration of ergot alkaloids in
551 mature plants infected with the Δ*velA* mutant showed no difference to the wild type,
552 correlating with the weak regulatory effect of VelA on their gene expression. The
553 chemical concentration of most indole diterpenes was significantly greater in Δ*velA*
554 mutant infected plants but this did not correlate with greater gene expression. It is
555 likely that the higher biomass of Δ*velA* mutant *E. festucae* in infected plants, even
556 led to the higher alkaloid levels. However, there is not always a strong correlation
557 of gene expression with alkaloid production, as also observed in Δ*sakA*, Δ*noxA* and
558 Δ*proA* mutant associations [20].

559 Most ergot alkaloids were detected at lower concentrations in Δ*laeA* mutant
560 infected plants and this was less significant for Δ*velA* mutant infected plants
561 compared to wild type. Despite this, this difference was greater for most of the
562 indole-diterpene alkaloids. Therefore, VelA and LaeA exert different influences on
563 alkaloid production in the *E. festucae*- perennial ryegrass association.

564 Of all the putative SSPs in *E. festucae*, 3.4%, 30% and 16.7% were
565 differentially expressed in IC Δ*velA*-WT, S Δ*velA*-WT and IP Δ*velA*-WT
566 comparisons, respectively. It seems VelA is a key regulator of SSPs expression in *E.*
567 *festucae* during its interaction with ryegrass. The greater numbers of differentially
568 expressed SSPs in infected seedlings and mature plants inoculated with the Δ*velA*
569 mutant, compared to in culture conditions, demonstrates their importance during the
570 infection process and survival. This has also been shown in other pathogenic or
571 symbiotic fungi (Reviewed in Presti et al 2015). Although there are more
572 differentially expressed SSPs in seedlings inoculated with the mutant compared to
573 mature infected plants a much broader range of fold changes were observed in
574 mature plants. This suggests condition-dependent regulatory roles of VelA for SSPs'
575 gene expression that possibly is a result of different mechanisms involved during the
576 fungal interaction under these two conditions. Possibly, VelA by regulating different
577 SSPs during the infection process and in mature infected plants positively regulates
578 SSPs that are required for establishing and maintaining symbiotic interaction, or
579 negatively regulates SSPs that lead to incompatible interactions. On the other hand,
580 6.2% and 16% of SSPs were differentially expressed in IC Δ*laeA*-WT and S Δ*laeA*-
581 WT, respectively. Compared to the *velA* related comparisons, it seems LaeA has

582 higher regulatory effects in culture and lower in seedlings compared to VelA. This
583 suggests different regulatory effects for these two related global regulators.

584 In conclusion, this study provides a significant understanding on the strong
585 regulatory effects of VelA on the *E. festucae* transcriptome profile during compatible
586 and incompatible interactions with ryegrass. These regulatory effects could explain
587 some of the observed phenotypes resulting from deleting *velA* in this fungus [11]. It
588 seems that during the interaction of *E. festucae* with ryegrass, VelA controls fungal
589 gene expression to supress or activate genes involved in either inducing or reducing
590 the hostplant response.

591 **Methods**

592 **Sample preparation**

593 Total RNA was extracted from samples comprising fungus grown in culture,
594 perennial ryegrass seedlings and mature perennial ryegrass plants (Table 1). For
595 culture conditions, *E. festucae* wild type and Δ*velA* mutant strains were grown for
596 two weeks on cellophane covered PDA medium in full darkness prior to harvest.
597 Seedlings were grown and harvested as previously described [37]. 7-10 d old
598 endophyte-free seedlings of perennial ryegrass (*L. perenne* 'Nui') were inoculated
599 with wild type and Δ*velA* mutant strains *E. festucae* (Generated previously [11, 70]),
600 which were grown for two weeks on PDA medium. Inoculated seedlings, grown for
601 two weeks under 16 h of 650 W/m^2 light and 8 h dark and after freezing, samples
602 from 4 cm upwards and 0.5 cm downwards from the meristem were collected for
603 RNA extraction and around 100 seedlings for each sample were pooled in three
604 replicates for each treatment.

605 For the mature plants, the top 4 cm of the newest mature blade of infected
606 plants with different strains were collected in three replicates (three mature plants
607 infected with the same strain) for each treatment.

608 RNA quality and quantity were determined using an Agilent 2100
609 Bioanalyzer (Agilent Technologies), Nanodrop Lite spectrophotometer (Thermo
610 scientific) and gel electrophoresis using a 1% agarose gel. RNA samples on dry ice
611 were sent to the Beijing Genomics Institute (BGI, Hong Kong) for sequencing and

612 2 µg of RNA sample used to prepare libraries by BGI standard method
613 (http://www.bgi.com/services/genomics/rna-seqtranscriptome/#tab-id-2). Samples
614 were sequenced in two lanes of an Illumina HiSeq4000 (paired end, 100-bp reads).

615 **RNA extraction and quantitative real-time RT-PCR analysis**

616 RNA extraction and complementary DNA (cDNA) were synthesised from 2
617 µg of RNA and Real time qPCR was performed with 1 µl of cDNA as described
618 previously [12] using primers that amplified target genes (Table S1). RNA samples
619 alone (no reverse transcription) and water-only (no template) controls were used to
620 detect gDNA and experimental contamination. Comparative ΔCt normalized to
621 gamma actin and 60S ribosomal protein L35 was used to calculate transcription
622 levels by using the geNorm algorithm automated in CFX manager software (Bio-
623 Rad).

624 **HiSeq results analysis**

625 HiSeq results analysis were done as described in Rahnama *et al.* (2019) [37] by
626 using a combination RNA-star version 2.5.0c [71], for mapping the HiSeq reads against
627 the genome dataset of *E. festucae* Fl1, with EdgeR package version 3.10.5 [72], for
628 counting the mapped genes. Fold changes and p-values were generated using Exact Tests
629 for differences between two groups of Negative-Binomial Counts.

630 Gene ontology (GO) and functional annotation analysis of differentially
631 expressed genes were performed as described in Rahnama *et al.* (2019) [37].

632 **General bioinformatics analyses**

633 Venn diagrams were drawn using BioVenn online software [73]. Volcano
634 plots were drawn in R statistical software environment version 3.2.1 [74].

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Authors' contributions

DJF, RDJ and MR designed the project. MR performed the experiments and drafted the manuscript. MR and PM performed bioinformatics. MR and RDJ revised the manuscript.

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Availability of data and materials

HiSeq Illumina sequencing included 36 raw sequence data sets that have been deposited into the NCBI SRA database with the BioProject ID PRJNA578737.

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Figure legends:

Fig. 1- Relative expression of *velA* and *laeA* genes measured by qRT-PCR. a) Relative expression of *velA* in wild type *E. festucae* at different times after inoculating seedlings (HPI: hours post inoculation; DPI: days post inoculation). b) Relative expression of laeA from some chosen conditions in vitro, in culture (PDA- D: potato dextrose agar under dark), seedlings (14 DPI- L: days post inoculation under light) and mature plants (old blade). Expression values were normalised to that of the gamma actin and 60S ribosomal protein L35. Primers used in the analysis are listed in Table S1. Bars represent standard error of the mean calculated from three biological replicates. Results were analysed using the MANOVA Tukey's test and statistically significant results are indicated with different letters.

Fig. 2- Percentage and distribution of differentially expressed genes in different comparisons and different conditions. a) The bar chart shows the percentage of DEGs that up or down-regulated in different comparisons. b) Venn diagrams show the common DEGs genes between different comparisons. c) Volcano plots show distribution of $\log_2$ of fold changes (logFC) and significantly ($-\log_{10}$ of FDR) in different comparisons. Black dots: FDR>0.05, Red dots: FDR<0.05, orange dots: logFC>1, green dots: FDR<0.05 & logFC>1.

Fig. 3- Expression changes of *E. festucae* alkaloid biosynthesis genes in different conditions. a) Expression changes of *E. festucae* alkaloid biosynthesis genes in different comparisons including S Δ*laeA*-WT, S Δ*velA*-WT, and IP Δ*velA*-WT. Dashed bars: fold changes that are statistically significant (FDR≤0.05), non-dashed bars: fold changes that are statistically non-

978 significant (FDR≥0.05). b) Relative expression levels of three representative genes of
979 ergovaline, lolitrem B and peramine biosynthetic genes in wild type, Δ*velA* and *ΔvelA/velA*
980 in three different conditions. Results were analysed using the MANOVA Tukey's and the
981 independent sample T-test and statistically significant results are indicated with same letter
982 are not significantly different.

983 **Fig. 4-** VelA regulates alkaloid production of *E. festucae* in ryegrasses plants. a) Alkaloids'
984 concentration in three months old ryegrasses infected with wild type and Δ*velA* mutant *E.*
985 *festucae*. Results were analysed using the independent sample T-test and statistically
986 significant results are indicated (***p<0.001, ** 0.05>p>0.001, * 0.1>p>0.05). b) The
987 differences of mean concertation of the wild type infected plants to the Δ*velA* mutant infected
988 plants and the mean concertation differences of wild type infected plants to Δ*laeA* mutant
989 infected plants.

990 **Fig. 5-** Transcriptomics profile of all secondary metabolism clusters in *Epichloë festucae*.
991 a) Heatmap of all genes in each secondary metabolism cluster. Grey colour cells showing
992 that in that condition none of the mutant or wild type are expressed. b) Percentage of DEGs
993 in the clusters with at least one DEGs.

994 **Fig. 6-** DEGs with putative SSP function. a) Number of DEGs, down or up regulated, in
995 each comparison with putative SSPs functions. b) Distribution of SSPs fold change in
996 different conditions, each point representing one SSP. c) Heat map showing the different
997 ranges of regulatory effects of VelA and LaeA on the expression of putative SSPs in
998 different conditions. d) Venn diagram shows the common SSPs between different
999 comparisons which significantly differentially expressed.

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Table 1- Different conditions and comparisons that used for the transcriptomics analysis. WT, wild type; D, dark; L, light; PRG, perennial ryegrass.

Conditions	**In culture (IC)**		**In seedling (S)**		**In planta (IP)**	
Host	PDA medium covered by cellophane		PRG seedlings		Mature infected PRG	
Light condition	D		8h L&16h D		8h L&16h D	
Time	14 d		14 dpi		3 month post inoculation	
***E. festucae* strains and comparisons**	WT	Δ*velA*	WT	Δ*velA*	WT	Δ*velA*

Table 2- DEGs with transporter activity in all different comparisons

Model	**Predicted function**	**Fold change**					
		IC Δ*laeA*-WT	**S Δ*laeA*-WT**	**IC Δ*velA*-WT**	**S Δ*velA*-WT**	**IP Δ*velA*-WT**	**Presence in core set**
EfM3.003420	Phospholipid transporter	-1.1	-1.2	-1.1	-1.1	**2.5**	No
EfM3.007320	Copper transport	1.4	1.6	1.2	**2.8**	1.9	No
EfM3.009680	ABC transporter	-1.9	-1.8	-1.9	-1.7	1.2	No
EfM3.009730	ABC transporter	-2.0	**-2.2**	-1.7	-1.8	**-2.1**	No
EfM3.012390	Nitrate transporter	1.6	1.0	1.3	1.0	**4.9**	No
EfM3.012760	Malic acid transporter	1.5	-1.7	-1.0	**-3.0**	-1.4	**Yes**
EfM3.014790	ABC transporter	1.2	-1.8	**-2.1**	-1.9	-1.4	No
EfM3.018210	Proline-specific permease	-1.1	1.1	-1.0	1.2	**-2.2**	No
EfM3.020140	Sulfate transporter	1.1	1.4	1.2	**2.5**	-1.7	No
EfM3.025050	Amino acid permease	1.4	1.3	1.1	**2.2**	1.1	No
EfM3.025350	Transporter	-1.3	-1.3	-1.7	1.2	-1.1	No
EfM3.027520	ABC transporter	1.1	1.7	1.2	**3.1**	**2.8**	No
EfM3.027540	ABC transporter	1.3	1.4	1.4	**2.5**	**2.8**	No
EfM3.027570	Peptide transporter	**2.6**	**3.1**	**3.0**	**9.9**	**12.5**	**Yes**
EfM3.032550	ABC transporter	1.2	1.3	1.1	-1.2	1.2	No
EfM3.035410	Carboxylic acid transporter	1.4	2.0	-1.1	-1.0	**5.1**	**Yes**
EfM3.039020	Ammonium transporter	1.0	**2.0**	1.0	**3.0**	**4.5**	No
EfM3.040210	phospholipid-translocating ATPase	-1.2	-1.1	-1.1	-1.1	**2.4**	No
EfM3.045520	hydrogen ion transmembrane transporter	3.4	**-2.4**	8.6	**-8.4**	**-4.0**	No
EfM3.047210	Purine permease	-1.1	1.2	-1.1	1.3	**2.1**	No
EfM3.055090	ABC multidrug transporter	1.1	-1.2	-1.1	**-2.7**	1.3	No

EfM3.056220	ABC multidrug transporter	1.2	**-4.8**	-1.1	**-4.2**	**-3.4**	**Yes**
EfM3.058970	Na+/H+ antiporter	1.0	-1.1	-1.1	-1.1	**-2.1**	No
EfM3.066900	xanthine/uracil permease	1.1	-1.6	1.4	**-2.9**	**-6.1**	**Yes**
EfM3.074200	transmembrane transporter	2.0	-1.3	**2.8**	-2.6	-1.6	No
EfM3.075680	transmembrane transporter	-1.8	-2.6	**-2.1**	-2.4	-2.4	No
EfM3.000930	Zinc ion transporter	1.1	**-2.8**	-1.1	-1.4	1.1	No
EfM3.005950	Amino-acid permease	1.4	**2.4**	-1.0	1.5	1.4	No
EfM3.029800	ABC multidrug transporter	**2.4**	1.1	1.1	-1.5	-1.1	No
EfM3.064820	Sugar transporter	-1.7	**-2.4**	-1.2	1.2	-1.2	No
EfM3.077030	Inositol transporter	1.3	**2.2**	1.2	1.4	1.1	No
EfM3.080140	Calcium transporter	-1.1	**-2.4**	-1.0	-1.6	1.8	No
EfM3.158840	Metal ion transporter	1.1	**2.0**	1.0	**2.0**	1.1	No

Table 3- DEGs with host cell wall degradation activity. Fold changes showed in bold are statistically significant (FDR≤0.05).

				Fold change				
Model	**CAZyme class**	**Predicted function**	**Target cell wall component**	**IC Δ*laeA*-WT**	**S Δ*laeA*-WT**	**IC Δ*velA*-WT**	**S Δ*velA*-WT**	**IP Δ*velA*-WT**
EfM3.049570	GH13	Alpha- glucosidase	Cellulose	1.0	**-2.1**	-1.2	-1.5	-1.2
EfM3.053990	GH5	Endoglucanase	Cellulose	1.5	**2.1**	-1.1	1.1	1.3
EfM3.005420	N/A	Exo-1,4-beta-xylosidase	Hemicellulose	1.1	**1.9**	-1.0	**2.3**	1.5
EfM3.040190	GH10	Endo-1,4-beta-xylanase	Hemicellulose	1.7	**3.3**	1.1	**3.9**	**83.5**
EfM3.037040	GH62	Glycosyl hydrolase	Hemicellulose	1.1	**4.3**	1.0	**4.3**	**69.6**
EfM3.008730	CE8	Pectin methylesterase	Pectin	-1.3	1.7	1.0	**2.0**	1.4
EfM3.008610	CE5	Cutinase	Cutin	**2.5**	2.9	1.2	-2.0	2.9
EfM3.005300	N/A	Cuticle-degrading protease	Cuticle	1.0	**-2.8**	1.0	**-5.1**	**-4.1**

Table 4- Differentially expressed genes engage in fungal cell wall composition. Fold changes showed in bold are statistically significant (FDR≤0.05).

			Fold change				
Model	**CAZyme class**	**Predicted function**	**IC Δ*laeA*-WT**	**S Δ*laeA*-WT**	**IC Δ*velA*-WT**	**S Δ*velA*-WT**	**IP Δ*velA*-WT**
EfM3.000810	CBM18	Chitinase	1.0	**-3.0**	1.0	**-3.3**	**-3.1**
EfM3.024310	GH18	Endochitinase B1	1.4	1.6	-1.1	**1.9**	**2.1**
EfM3.049120	GT2	Chitin synthase	-1.3	-1.4	**2.0**	1.3	-1.4
EfM3.056450	CBM43	Glucanosyl transferase	-1.2	-1.2	-1.0	-1.2	**2.1**
EfM3.056810	GH64	Glucan endo-1,3-beta-glucosidase	1.1	1.1	-1.1	1.0	**-2.2**
EfM3.078790	N/A	Cell wall protein SED1	1.1	**-3.4**	1.1	**-2.5**	-1.8
EfM3.054000	N/A	Related to cell wall glycoprotein	-1.7	1.1	1.1	**2.3**	1.9
EfM3.034340	N/A	Cell wall glycoprotein	-1.2	**-3.4**	1.4	-1.9	**-3.7**

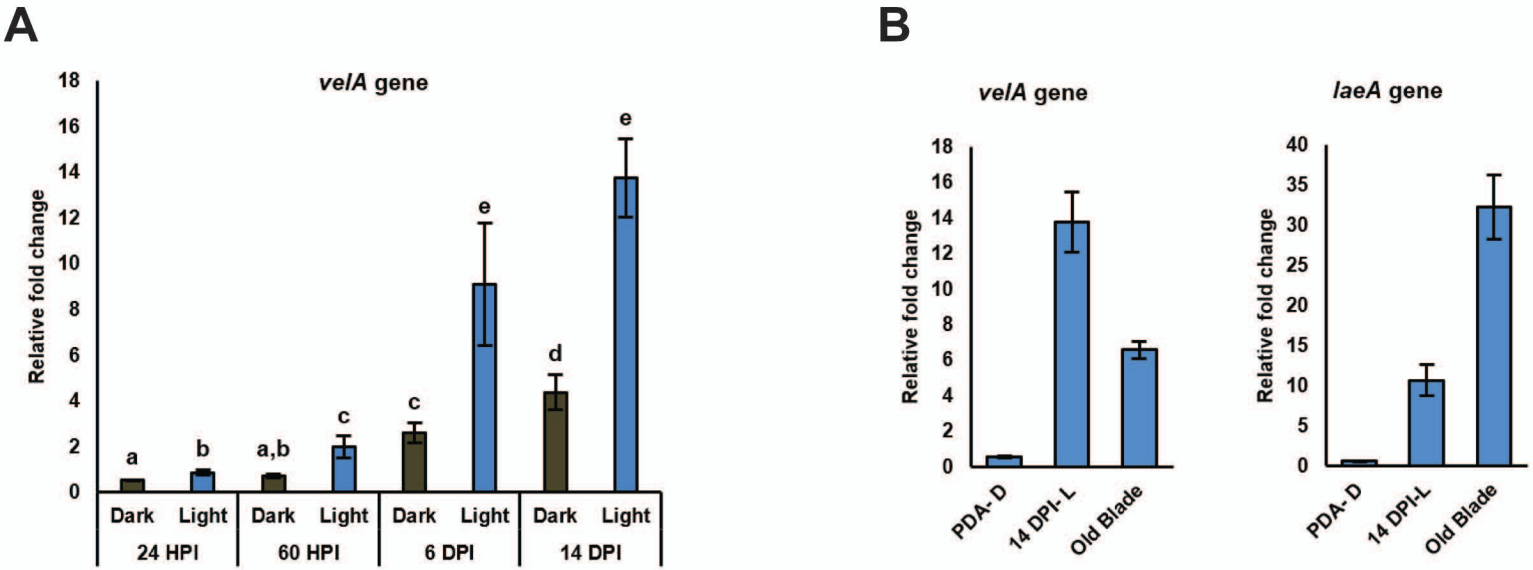


Fig. 1_ Rahnama et al.

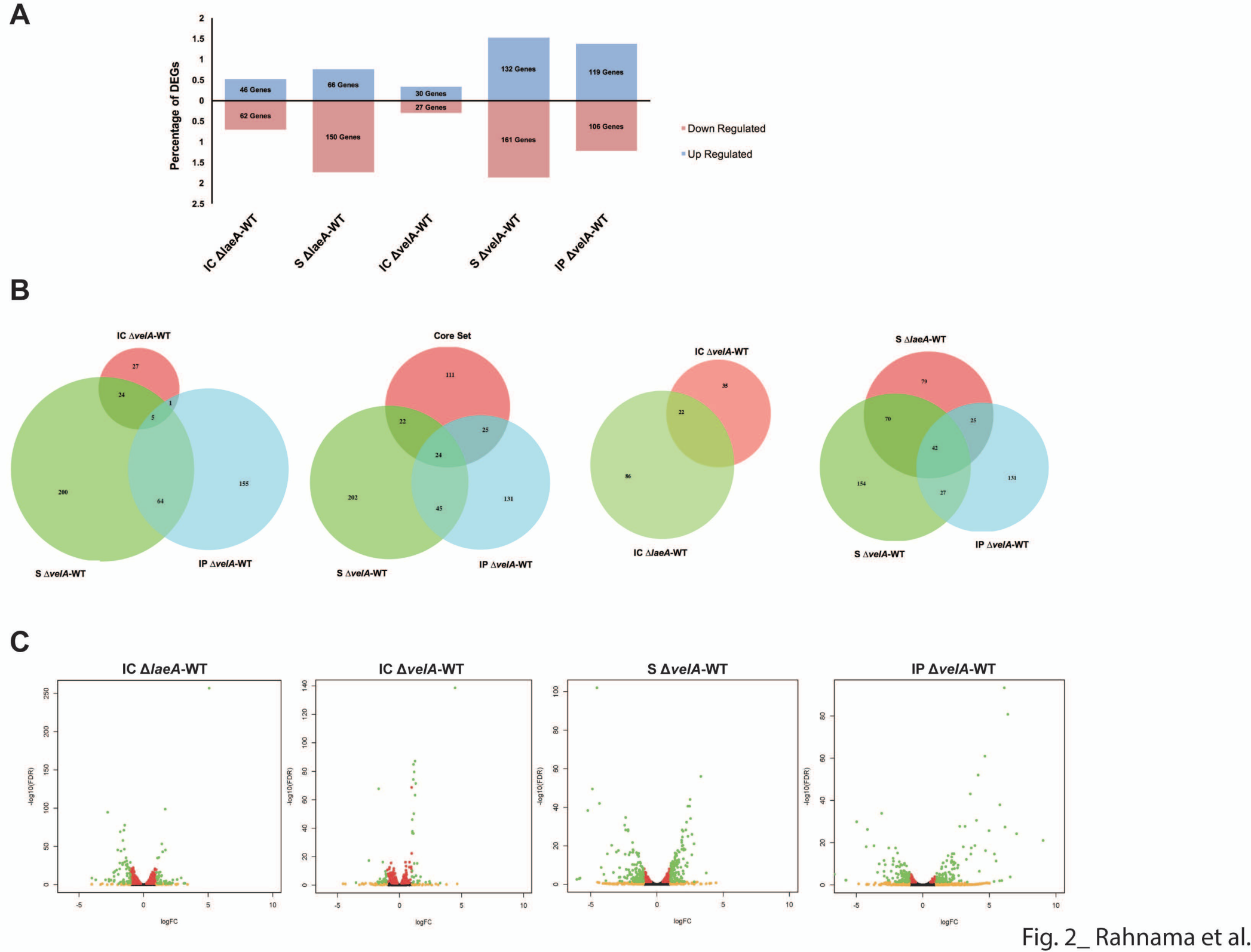


Fig. 2_ Rahnama et al.

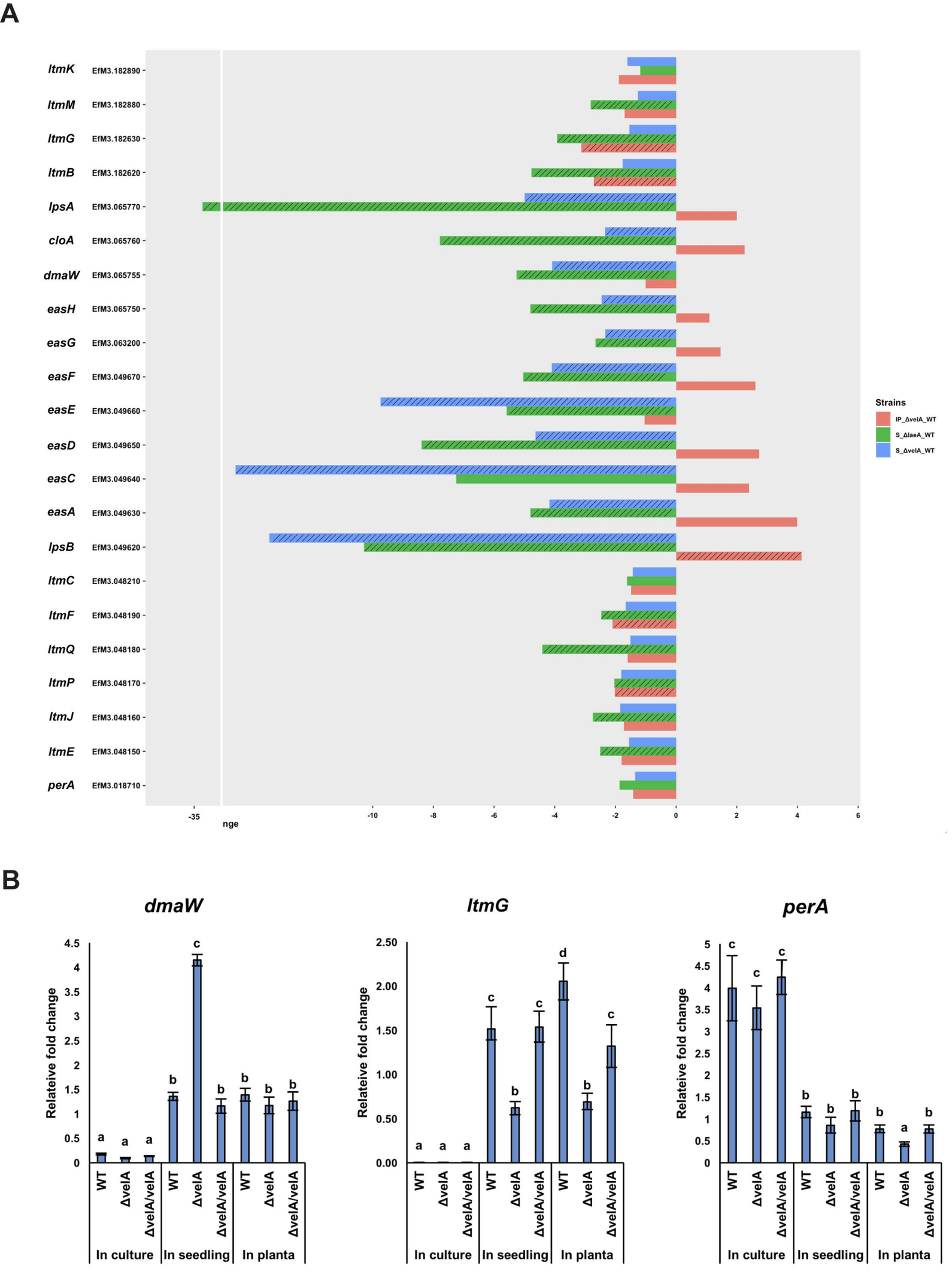
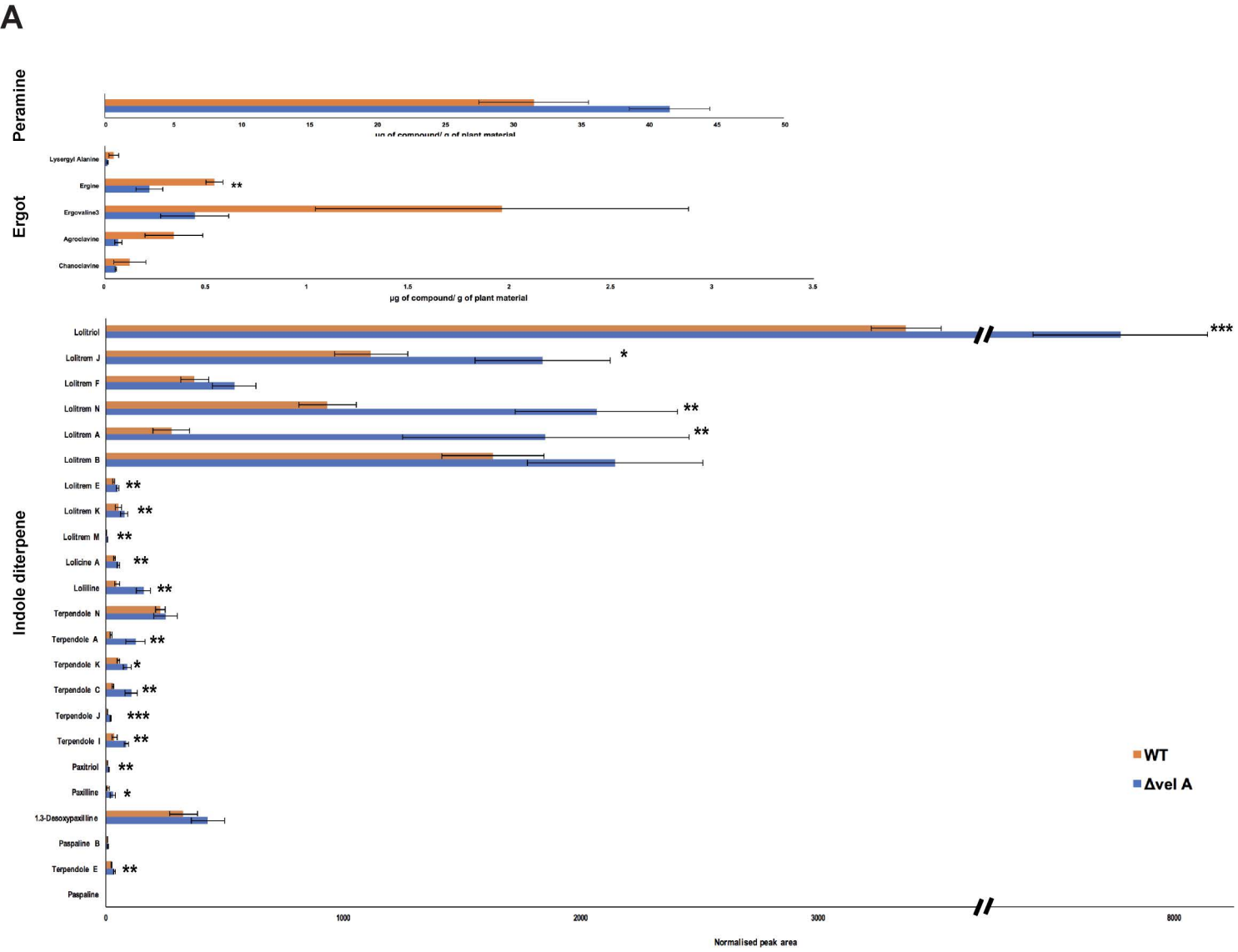


Fig. 3_ Rahnama et al.



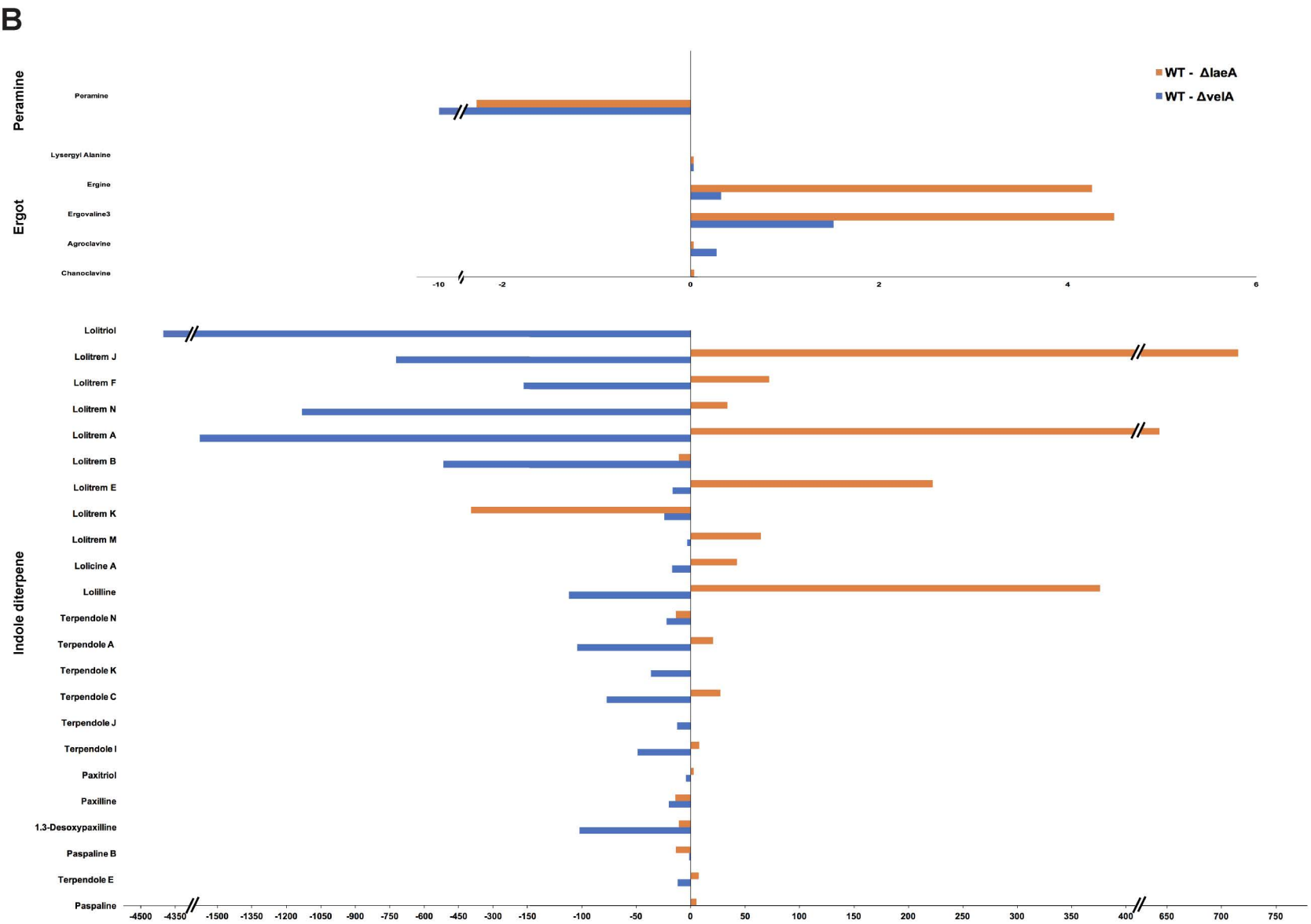
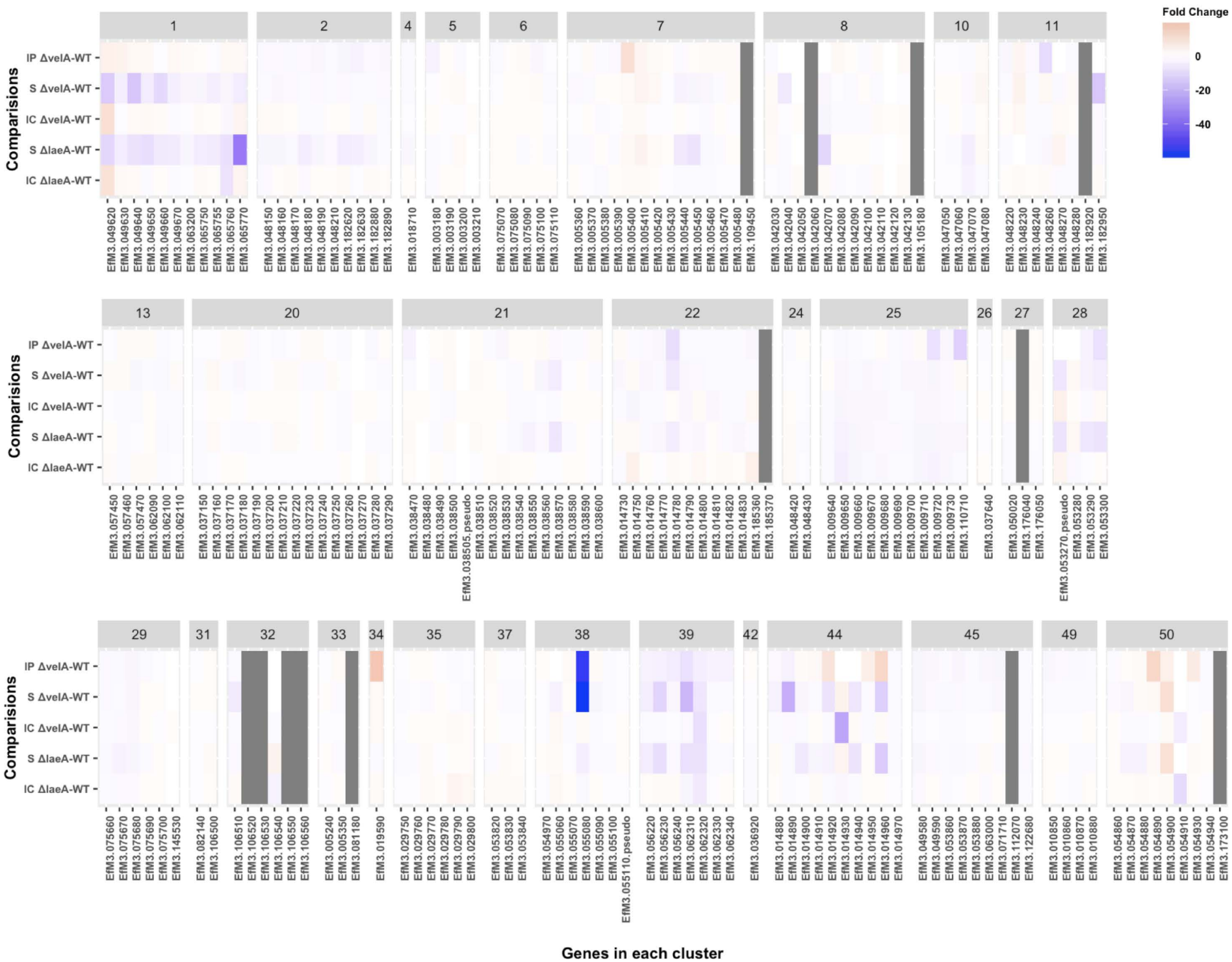


Fig. 4_ Rahnama et al.

A



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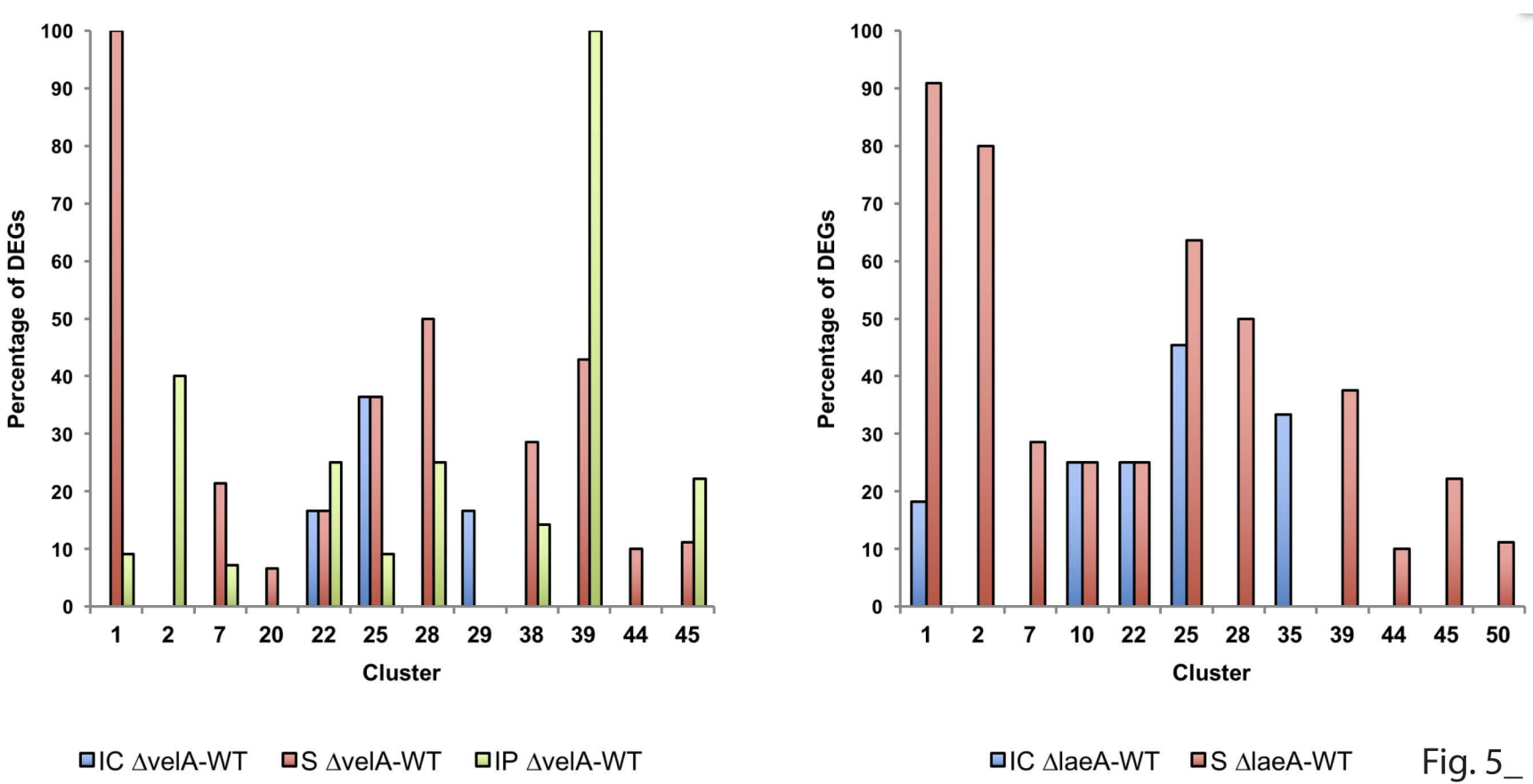

Fig. 5_ Rahnama et al.

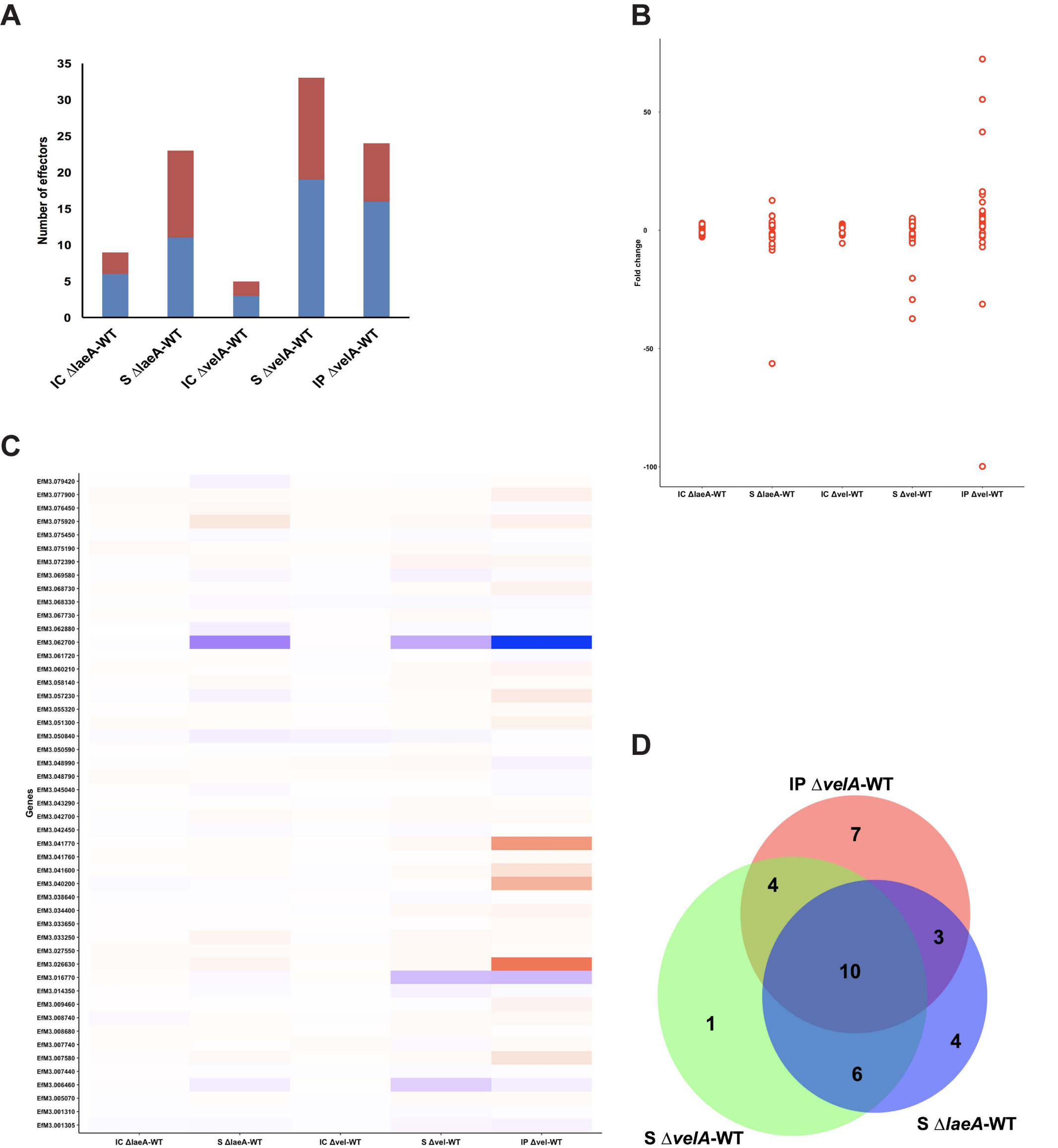

Fig. 6_ Rahnama et al.