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In planta transcriptomics reveals conflicts between pattern-triggered immunity and the AlgU sigma factor regulon

Haibi Wang[1], Amelia Lovelace[2], Amy Smith[1], Brian H. Kvitko[1,3]*

1 Department of Plant Pathology, University of Georgia, Athens GA

2 The Sainsbury Laboratory, Norwich Research Park, Norwich, NR4 7UH, UK

3 The Plant Center, University of Georgia, Athens GA

HW contributed to data analysis and writing, AL contributed to sequencing data processing, AS contributed to RNA sample preparation and sequencing, BK oversaw the project.

*Corresponding author: B.H.Kvitko; E-mail: bkvitko@uga.edu

Keywords: *Arabidopsis thaliana*, *Pseudomonas syringae*, plant immunity, PTI, *algU*, T3E, motility, transcriptome

Abstract

In previous work, we determined the transcriptomic impacts of flg22 pre-induced Pattern Triggered Immunity (PTI) in *Arabidopsis thaliana* on the pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto*). During PTI exposure we observed expression patterns in *Pto* reminiscent of those previously observed in a *Pto algU* mutant. AlgU is a conserved extracytoplasmic function sigma factor which has been observed to regulate over 950 genes in *Pto in vitro*. We sought to identify the AlgU regulon *in planta*.and which PTI-regulated genes overlapped with AlgU-regulated genes. In this study, we analyzed transcriptomic data from RNA-sequencing to identify the AlgU *in planta* regulon and its relationship with PTI. Our results showed that approximately 224 genes are induced by AlgU, while another 154 genes are downregulated by AlgU in *Arabidopsis* during early infection. Both stress response and virulence-associated genes were induced by AlgU, while the flagellar motility genes are downregulated by AlgU. Under the pre-induced PTI condition, more than half of these AlgU-regulated genes have lost induction/suppression in contrast to naïve plants, and almost all function groups regulated by AlgU were affected by PTI.

Key words: *Arabidopsis thaliana*, *Pseudomonas syringae*, plant immunity, PTI, *algU*, T3E, motility, transcriptome

40   The plant cell produces surface receptors that allow it to recognize Pathogen

41   Associated Molecular Patterns (PAMPs) and trigger Pattern Triggered Immunity

42   (PTI). In the model organism *Arabidopsis thaliana* Col-0*,* PTI induction is

43   associated with a series of responses including a rapid ROS (reactive oxygen

44   species) burst, induction of defensive hormone biosynthesis pathways, changes

45   in apoplast and cell wall composition, and increased expression of pathogen-

46   related receptors (DeFalco and Zipfel 2021; Jwa and Hwang 2017; Ngou et al.

47   2021; Luna et al. 2011). The flagellin-derived peptide flg22 is a synthetic PAMP

48   commonly used to activate PTI in laboratory settings. Upon recognizing flg22, the

49   *Arabidopsis* FLS2 receptor initiates PTI responses, reducing proliferation of the

50   model pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto*) (Zipfel et al.

51   2004). An activated PTI response also restricts Type III effector delivery by *Pto*

52   and reduces expression of the *P. syringae* virulence regulon (Crabill et al. 2010;

53   Lovelace et al. 2018; O'Malley and Anderson 2021; Nobori et al. 2018).

54   Upon transitioning into the leaf apoplast *Pto* has the capacity to perceive and

55   respond to niche-specific cues to reprogram gene expression and express plant

56   virulence factors. Extracytoplasmic function (ECF) sigma factors are a common

57   tool used by bacteria to achieve such reprogramming. One ECF sigma factor,

58   AlgU (RpoE), is known to regulate hundreds of genes involved in metabolism,

59   motility, stress tolerance, and virulence in *Pto* (Markel et al. 2016). Deletion of the

60   *algU* gene or the *algUmucAB* operon, which includes the anti-sigma factors of

61   AlgU, reduces *Pto* growth in tomato and *Arabidopsis* seedlings (Markel et al.

62   2016; Ishiga et al. 2018).

63   Previous studies have identified *Pto* genes with altered regulation during

64   exposure to pre-induced PTI in *Arabidopsis* including motility related genes,

65   osmotic stress response genes, and alginate synthesis genes (Lovelace et al.

66   2018; Nobori et al. 2018). Although these genes overlap with the known *in vitro*

67   AlgU regulon, it is not known to what degree the AlgU *in planta* regulon (AlgU-

68   regulated genes in the plant-niche) resembles the *in vitro* AlgU regulon (AlgU-

69   regulated genes in growth media), or how PTI modifies the AlgU *in planta*

70   regulon. In this study, we identified the *Pto* AlgU regulon during early

71   interactions with the *Arabidopsis* adult leaf apoplast, and in addition show that

72   pre-induced PTI intervenes against the induction or suppression of more than

73   half of these AlgU-regulated genes.

74

75   **Results**

76   <u>Transcriptomic differences between *Pto* WT and *ΔalgU* during early *Arabidopsis*</u>

77   <u>infection reveals the AlgU *in planta* regulon</u>

78   In order to identify the AlgU *in planta* regulon, we infiltrated *Arabidopsis* with

79   either wild type (WT) or *ΔalgUmucAB* (*ΔalgU*) *Pto*, and collected total RNA

80   samples at 5 hpi (hours post inoculation) for RNA-seq using previously

81   established techniques (Lovelace et al. 2018). This time point was chosen to be

82   consistent with said study. After plant host and bacterial rRNA depletion, samples

83   were sequenced by RNA-Seq. The resulting reads were cleaned and mapped to

84   the *Pto* reference genomes and differentially-expressed genes (DEGs) were

85 identified for both strains. By using a cutoff at 1.3-fold change in expression and

86 false discovery rate less than 5%, we found that 224 genes were upregulated,

87 while 154 genes were down regulated in the WT background compared to the

88 *ΔalgU* background (Fig. 1A and Table S1 and S2). To confirm our observations,

89 we validated the expression change patterns of genes belong to function groups

90 of interest including alginate production (*algD*), osmotic stress response (*opuCA*),

91 motility (*fliD*), and virulence (*avrPto1*) by Reverse Transcription-quantitative PCR

92 (RT-qPCR) (Fig. S1). It is worth noting that since some of these genes are

93 encoded in polycistronic operons, the number of different transcripts regulated by

94 AlgU is smaller than the number of the identified genes.

95 For the AlgU upregulated *in planta* regulon, we were able to identify genes

96 involved in osmotic stress response, oxidative stress response, alginate

97 synthesis and export, cell shape and division pathways, DNA repair pathways,

98 Type II Secretion System structural genes, and macromolecule metabolism and

99 energy production (including lipid metabolism, amino acid metabolism, nucleotide

100 metabolism, tRNA synthesis, ribosomal protein, transporter and permease

101 genes). We also found that Type III Secretion System effectors and transcription

102 factors are part of the AlgU regulon (Table 1).

103 For the AlgU downregulated *in planta* regulon, we identified genes involved in

104 flagellar assembly, chemotaxis, signal transduction pathways, Type IV

105 conjugation pilus assembly, DNA modification pathways, and macromolecule

106 metabolism and energy production (including lipid metabolism, amino acid

107 metabolism, nucleotide metabolism, protein translation and degradation, and

108   transporter genes). We also identified GGDEF/EAL domain containing proteins

109   and transcription factors among downregulated genes (Table 2).

110

111   <u>Pre-induced PTI response intervenes against the AlgU *in planta* regulon</u>

112   It was previously observed that the *Pto* genes affected by pre-induced PTI

113   include those that are regulated by AlgU. To further identify the portion of the

114   AlgU *in planta* regulon that is affected by PTI, we compared the AlgU *in planta*

115   regulon to the list of genes that are affected by PTI (Lovelace et al. 2018). We

116   found that 124 (55%) genes from the AlgU upregulated *in planta* regulon, and 91

117   (59%) genes from the AlgU downregulated *in planta* regulon are intervened

118   against by the pre-induced PTI (Fig. 1B), which involves all the functional groups

119   except iron-sulfur cluster related genes (Table 3, S3, and S4). The majority of the

120   remaining AlgU *in planta* regulon genes remain the similar expression level under

121   pre-induced PTI conditions compare to naïve conditions, namely 86 (38%) genes

122   from the AlgU upregulated *in planta* regulon, 63 (41%) from the AlgU

123   downregulated *in planta* regulon. In contrast, 14 (6%) genes in the AlgU

124   upregulated *in planta* regulon are further upregulated under pre-induced PTI

125   condition, and none of the AlgU-suppressed *in planta* regulon genes is further

126   downregulated (Table S5). Taken together, these numbers suggest that the pre-

127   induced PTI response and AlgU *in planta* regulon are in conflict.

128

129 ### Stress responsive genes are AlgU induced and intervened against by PTI at 5

130 ### hpi

131 AlgU regulates the expression of osmotic and oxidative stress response genes in

132 different bacteria (Wang et al. 2021). Alginate, a secreted polysaccharide, is also

133 generally considered to shield bacteria from external stressors (Chang et al.

134 2007; Keith et al. 2003). We analyzed the relationship between AlgU and these

135 stress tolerance related genes *in planta*. Glycine betaine transporter genes

136 (*opuCABCD, cbcVWX*)(Chen and Beattie 2007; Chen et al. 2010) were

137 dependent on AlgU for induction, and are PTI inhibited (Fig. 2A). However, for

138 compatible solute synthesis genes, even though their induction is AlgU

139 dependent, their expression was not inhibited by the pre-induced PTI at 5 hpi.

140 The oxidative stress response genes (according to Yu 2014) *trx-2*(PSPTO_5243),

141 *sodB* (PSPTO_4363), and a glutaredoxin domain protein (PSPTO_4161) are

142 also part of the AlgU *in planta* regulon that are intervened against by PTI (Table

143 S1 and S3). Alginate synthesis genes showed a similar trend to the glycine

144 betaine transporter genes, they are induced by AlgU, and intervened against by

145 pre-induced PTI (Fig. 2B). These results suggest that at 5 hpi, the bacterial cells

146 activate genes related to osmotic stress response and alginate production with

147 the help of AlgU, while pre-induced PTI can have a negative effect on the

148 induction, which likely reduces the bacterial capability for tolerating stresses.

149

150 ### Multiple secretion system-associated genes are AlgU induced and intervened

151 ### against by PTI at 5hpi

152   The Type III Secretion System (T3SS) is a needle-like structure that secrets

153   unfolded effector proteins, and is required for *Pto* virulence (O'Malley and

154   Anderson 2021; Lindgren et al. 1986). Type III effectors (T3Es) are delivered into

155   the host cells and are critical for suppressing PTI responses in naïve plants.

156   Genes encoding T3Es are distributed at more than ten locations in the *Pto*

157   genome (Cunnac et al. 2011; Wei et al. 2015). Our data showed that AlgU

158   enhances induction of 17 out of the 36 T3Es at 5 hpi, and these same genes are

159   suppressed by pre-induced PTI (Fig. 3). However, T3SS structural genes are not

160   significantly AlgU induced, even though they are suppressed by pre-induced PTI

161   (Fig. S2).

162   Another secretion system that may also play a role in PTI response manipulation,

163   the Type II Secretion System (T2SS), transports folded proteins from periplasm

164   to the extracellular space. Our data showed that ten T2SS structural component

165   genes are AlgU induced at 5 hpi, and *gspD, gspN*, and *gspM* are suppressed in

166   the pre-induced PTI environment (Fig. 4). Interestingly, *gspDNM* are the three

167   last genes within the cluster, and the first several genes in the same cluster are

168   not significantly PTI-suppressed. One explanation is that these genes may be

169   transcribed from separate promoters.

170   Coronatine is a plant hormone mimic and virulence factor produced by *Pto*. It

171   was shown previously that AlgU may play a role in coronatine synthesis gene

172   regulation (Ishiga et al. 2018). Our data showed that, at 5 hpi *in planta*, AlgU

173   significantly upregulated the expression of only two coronatine-associated genes:

174   *hopAQ1,* which may be co-transcribed with the regulator for coronafacic acid

175 (CFA) and coronamic acid synthesis (CMA) called *corRS*, but is not known to

176 directly relate to coronatine synthesis, and *cmaL*, which is encoded away from

177 the primary biosynthetic CFA and CMA operons (Worley et al. 2013)(Fig. S3).

178 Regardless of the limited involvement of AlgU in coronatine gene regulation,

179 most of the coronatine biosynthesis genes are inhibited by pre-induced PTI.

180

181 Flagellar motility-related genes are AlgU suppressed and intervened against by

182 PTI at 5 hpi

183 In rich media, AlgU suppresses swimming motility by lowering the expression of

184 several flagella-related genes including the *fliC* flagellin gene. Reduced flagellin

185 expression can reduce FLS2-mediated responses in tobacco and tomato (Bao et

186 al. 2020). We sought to answer if AlgU regulates motility related genes in

187 *Arabidopsis*. The 60+ flagellar assembly and chemotaxis related genes in *Pto* are

188 organized as a large single gene cluster on the chromosome. Studies in *P.*

189 *aeruginosa*, which possesses a syntenous cluster, suggested that these genes

190 can be organized into four classes based on their expression hierarchy

191 (Dasgupta et al. 2003) (Fig. 5A) . Our data showed that the presence of AlgU in

192 the WT background reduced expression of most of the four classes of genes at 5

193 hpi, and PTI-exposure increased the expression of all these genes (Fig. 5B).

194 Interestingly, several genes proposed to be involved in swarming motility showed

195 the same trend as the four classes of flagellar genes (Fig. 5C). Syringafactin and

196 3-(3-hydroxyalkanoyloxy) alkanoic acid (HAA) are two surfactants, and their

197 production is directly related to swarming motility (Nogales et al. 2015; Burch et

198 al. 2012). The syringafactin production gene *syfA* and its transcription regulator

199 *syrR(*also called *syfR)*, and the HAA production gene *rhlA* homolog *phaG-1*

200 (PSPTO_3299) are all downregulated by AlgU and upregulated under pre-

201 induced PTI conditions. In addition, the two genes *fgt1* and *fgt2*

202 (PSPTO_1946/1947), which contribute to flagellar glycosylation and swarming

203 motility (Taguchi et al. 2006), are similarly regulated. These results suggest that

204 AlgU promotes the cells to enter a non-motile state at 5 hpi, while pre-induce PTI

205 promotes the cells to keep expressing motility related genes.

206

207 <u>Multiple transcription factors have altered regulation *in planta* in the absence of</u>

208 <u>AlgU, and are affected against by pre-induced PTI at 5 hpi</u>

209 A previous study using ChIP-Seq has shown that many of the AlgU-regulated

210 genes have no AlgU binding near their promoter region (Markel et al. 2016). One

211 explanation is that AlgU affects the expression of these genes indirectly through

212 other transcriptional regulators, either by direct induction, or through feedbacks.

213 Our data showed that 19 genes with known or predicted transcriptional regulatory

214 function, including *hrpL*, *amrZ*, and *fur* are induced when AlgU is present, and

215 another 13 transcriptional regulators including *fleQ* and *syrR* are suppressed in

216 the presence of AlgU at 5 hpi (Fig. 6). Pre-induced PTI significantly affected 9 of

217 these induced genes and 10 of these suppressed genes. Interestingly, *amrZ*

218 showed a strong AlgU dependence for induction, but it is induced under PTI

219 condition rather than suppressed, dissimilar to the others.

220

Discussion

222 In this study, we compared transcriptomes from *P. syringae* pv. *tomato* DC3000

223 WT and *ΔalgU* strains, either at 5 hpi in four-week-old *Arabidopsis thaliana* Col-0

224 with or without flg22 pre-treatment, or in KB media, to find AlgU-regulated genes

225 specific to the apoplastic niche, which we termed "AlgU *in planta* regulon". We

226 then compared the transcriptome profile of these genes under pre-induced PTI

227 and naïve conditions and determined that PTI affects more than half of AlgU-

228 regulated genes, and that PTI affects almost all function groups regulated by

229 AlgU. Our result suggests that AlgU and PTI-mediated regulation may be in

230 conflict.

231 AlgU *in planta* regulon is smaller from that was observed *in vitro*

232 The number of genes in the AlgU *in planta* regulon is smaller than the published

233 AlgU *in vitro* regulon (Markel et al. 2016), which was generated using an AlgU

234 over-producing strain in King's B media. Our study looked at native expression,

235 but not over-expression, which may account for the difference between the two

236 results. The smaller size of the AlgU *in planta* has also been observed from a

237 previous study, which looked at the AlgU regulon under different *in vitro* stressors

238 or in association with plant hosts and showed that subsets of many transcription

239 factor regulons are condition specific (Yu et al. 2014). It is possible that in the

240 plant niche, a subset of the transcriptional factors in the AlgU regulon can

241 incorporate condition specific signals. Another explanation for the environment-

242   specific regulation is that AlgU regulon genes are co-regulated by other

243   transcription factors. Previous studies and our data have shown that the AlgU

244   regulon overlaps with other transcription regulators such as the sigma factors

245   RpoS, RpoN, HrpL, (Markel et al. 2016; Yu et al. 2014), small RNA regulators

246   RsmA2/A3 (Liu et al. 2021), the Fur iron homeostasis regulator (Butcher et al.

247   2011; Nobori et al. 2018), and the two-component system CvsSR (Fishman et al.

248   2018).

249

250   <u>Secretion systems</u>

251   Consistent with the AlgU *in vitro* regulon, we have identified multiple T3Es as part

252   of the AlgU *in planta* regulon. However, we could not find common regulatory or

253   functional features among these T3Es to explain why only a specific subset of

254   effectors are AlgU regulated. These T3E are from different clusters on the

255   genome, each has a different identified function in the plant cell, and each

256   interact with different plant proteins. Some T3Es targets both PTI and Effector

257   Triggered Immunity (ETI) pathways (HopAD1 (Wei et al. 2015) and HopU1

258   (Nicaise et al. 2013; Fu et al. 2007)). Some T3Es are associated with

259   cytoskeleton (HopG1 (Shimono et al. 2016; Block et al. 2010) and HopE1 (Guo

260   et al. 2016)). Some T3Es are associated with plant membrane proteins (HopF2

261   (Zhou et al. 2014) and HopAF1 (Washington et al. 2016)). The only shared

262   feature of these T3Es is that they suppress PTI. However, PTI suppression is a

263   common feature of T3Es and many of them are not a part of the AlgU *in planta*

264   regulon. The sigma factor HrpL, which regulates the expression of T3SS related

265 genes, was identified as an AlgU *in planta* regulated gene, similarly to the

266 observation from previous studies (Markel et al. 2016; Yu et al. 2014). It was

267 previously shown that *hrpL* is downregulated by PTI compared to naïve plants at

268 1 hpi and 3 hpi during infection, but is not significantly differentially regulated at 5

269 hpi, (Lovelace et al. 2018).

270 Unlike the T3SS, the importance of T2SS in the host-pathogen relationship has

271 been overlooked in *Pto,* which is a hemibiotrophic pathogen. In agreement with

272 the previously published AlgU regulon in growth media (Markel et al. 2016), our

273 data also showed that T2SS structural genes are upregulated by AlgU *in planta,*

274 and these genes are PTI suppressed. T2SS is a part of the general secretion

275 pathway (GSP) and transports folded proteins across the outer membrane. Even

276 though it was shown that a significant reduction in disease development

277 happened in both *ΔgspD* and *ΔgspE* background (Bronstein et al. 2005), most

278 research regarding to virulence factor secretion in *Pto* focused on the T3SS. In

279 contrast to *Pto*, T2SS is better understood in several other pathogens. In the

280 necrotrophic pathogens *Pectobacterium atrosepticum* and *Dickeya dadantii,*

281 T2SS is important for virulence, ROS tolerance (Charkowski et al. 2012; Liu et al.

282 2019), and promotes commensal bacterial growth via secretion of pectate lyases

283 and other cell-wall-degrading enzymes (Yamazaki et al. 2011). Similarly, in the

284 root commensal bacteria *Dyella japonica,* T2SS plays a role in PTI suppression

285 (Teixeira et al. 2021). In the human pathogen *P. aeruginosa*, T2SS is involved in

286 biofilm formation (Lewenza et al. 2017) and toxin secretion (Swietnicki et al.

287 2019). On the other hand, even though our data showed that T2SS is AlgU

288 induced *in planta* and is PTI-suppressed, we do not know which proteins are

289 T2SS substrates, because the substrate recognition signal is not universal and

290 cannot be easily predicted (Naskar et al. 2021; Pineau et al. 2014). The two

291 known substrates PlcA1 (PSPTO_3648) and PlcA2 (PSPTO_B0005) (Bronstein

292 et al. 2005) are neither significantly induced by AlgU nor affected by pre-induced

293 PTI (data not shown). Based on research from other bacteria, the substrates

294 secreted by T2SS in *Pto* may include lipases, proteases, phosphatases, and

295 more (Tilley et al. 2014; Urusova et al. 2019; Putker et al. 2013).

296 Interestingly, in contrast to a previous study which identified T6SS genes as a

297 part of the *P. syringae* pv. *syringae* B728a AlgU regulon in the bean apoplast (Yu

298 et al. 2014), our result did not identify any T6SS genes. There are two copies of

299 T6SS genes, the first copy HSI-I coded by genes PSPTO_2538-2554, and the

300 second copy HSI-II coded by PSPTO_5415-5438 (Sarris et al. 2010). Both

301 copies include both structural genes and secreted factors. Our data did not

302 identify any of these genes as part of the AlgU *in planta* regulon (data not shown).

303 This is similar to the previous observation that *P. syringae* B728a and *Pto* have

304 different T6SS regulation *in vitro* (Freeman 2013).

305

306 <u>Motility genes</u>

307 It is interesting that we saw both swimming and swarming motility/surfactant

308 related genes are suppressed by AlgU at 5 hpi, and are upregulated under the

309 pre-induced PTI condition. This suggests that in addition to the previously

310 proposed role for AlgU in immunity evasion, *Pto* may favor a nonmotile lifestyle in

311 naïve plants. The enhanced expression of flagellar genes in the absence of AlgU

312 has been shown to enhance immunity detection (Bao et al. 2020). It is interesting

313 that exposure to PTI-conditions results in a similar pattern of enhanced flagellar

314 gene expression by *Pto* which would presumably also enhance immune

315 detection by the host. It is unclear whether this is a maladaptive response by the

316 bacteria that favors the host or if PTI exposure signals are perceived by the

317 bacteria as repellants resulting in a motility-driven avoidance response.

318 According to the flagellar gene regulatory hierarchy, the two transcription factors

319 FleQ and FliA belong to class I. FleQ activates class II genes, which include the

320 cytoplasmic structural genes and the regulator FleR. FleR then activates class III

321 genes, which include the structural genes localized at the cell wall, outer

322 membrane, and extracellular space. FliA activates class IV genes, which include

323 flagellin, motor genes and chemotaxis genes (Dasgupta et al. 2003) (Fig. 5A).

324 Interestingly, we observed two unexpected patterns from the RNA-seq data. First,

325 even though PTI universally activated all these genes, the first gene in all FleQ

326 and FleR induced operons showed distinctive AlgU inducibility in contrast to the

327 other genes, as they are slightly induced while the following genes are

328 downregulated by AlgU. Additionally, FleQ was hypothesized to suppresses the

329 expression of the syringafactin regulator *syrR* (Nogales et al. 2015), but they both

330 were suppressed by AlgU according to data from this study. These observations

331 suggest that motility genes are not always co-regulated, and different regulatory

332 mechanisms may exist. Second, *fliA* expression level was not significantly

333   increased in the absence of AlgU, but most of FliA-regulated genes increased

334   expression regardless. This may be because the change in expression level in

335   the transcription regulator is not in proportion to the change in its regulated genes.

336   Surprisingly, our RNA-seq data did not show a significant increase in the

337   expression of *fliC* (Class IV) in the strain without *algU*, which is contradictory to

338   the previously reported results based on RT-qPCR using tomato as the host at

339   6hpi (Bao et al. 2020). Instead, our data agrees with data from another group that

340   also used *Arabidopsis* as the host plant (Ishiga et al. 2018). Whether the

341   difference in *fliC* expression is due to differences in the host environment or other

342   experimental factors remains an open question.

343   <u>Pre-induced PTI and AlgU in conflict</u>

344   Overall, our data indicates that the patterns of induction or suppression for AlgU-

345   regulated genes *in planta* are generally reversed during PTI exposure. At this

346   point we are unable to conclude whether PTI-associated responses interfere with

347   AlgU-mediated regulation directly or indirectly. The amount of free AlgU in the

348   cell is post-translationally regulated by the anti-sigma factors MucA and MucB,

349   which is regulated through the regulated intramembrane proteolysis (RIP)

350   pathway. It was previously shown in *Arabidopsis* that two secreted plant

351   proteases, SAP1 and SAP2, are induced during PTI and that these two

352   proteases degraded the RIP pathway-associated protease MucD (Wang et al.

353   2019). However, in *P. syringae* and *P. aeruginosa,* a *ΔmucD* strain has a hyper-

354   activated AlgU phenotype (Wang et al. 2019)(Damron and Yu 2011) while we

355   have observed that PTI exposure has the opposite effect on AlgU-regulated

356 genes. It is possible that AlgU is released via SAP1/SAP2 degradation of MucD

357 and overactivation of the RIP cascade, but other PTI responses ultimately

358 intervene against the AlgU regulon possibly through indirect overlapping

359 regulators. The transcription factor AmrZ (PSPTO_1847), which was shown to

360 have an AlgU binding site at its promoter region (Markel et al. 2016), is strongly

361 dependent on AlgU for expression *in planta* (Fig. 6). AmrZ is one of the 14 AlgU

362 upregulated genes that are also upregulated in PTI-activated plants compared to

363 naïve plants (Table S5), supporting the possibility that AlgU is activated during

364 PTI exposure. However, our data showed that these 14 genes make up less than

365 4% of the total AlgU *in planta* regulon, whether AlgU is activated and acted

366 against, or if AlgU activity is directly suppressed by PTI remains a question. The

367 specific cues within the apoplast that are perceived to induce the AlgU regulon

368 are still unknown. Determining what signals bacteria perceive in the apoplast to

369 drive AlgU activation and whether those signals are modified or masked during

370 PTI will be critical to understanding how this conserved bacterial sigma factor

371 interacts with this ancient form of plant immunity.

372

373 **Material and methods**

374 <u>Plant material</u>

375 *Arabidopsis thaliana* Col-0 seeds were sown in SunGrow Professional growing

376 potting mix in 3.5-inch square pots, stratified for one day at 4°C in darkness, then

377 moved to Conviron A1000 growth chamber with settings of 14 hour day, 23°C, 70

378   µmol light. After two weeks, the pots were thinned to 4 plants per pot. After four

379   weeks, the pots were moved to a growth room with settings of 12 hour day, 23°C.

380

381   <u>RNA sample preparation and sequencing</u>

382   The same procedure as described in a previous publication was used (Lovelace

383   et al. 2018). At four to five weeks' age, the four largest leaves of each plant were

384   treated with flg22 or mock solution. Flg22 peptide (GenScript RP19986) stock

385   solution was made by dissolving in DMSO to final concentration at 1mM. A 1000x

386   dilution of the flg22 stock solution in water was syringe infiltrated to induce PTI,

387   and a 1000x dilution of DMSO was used as the mock treatment for naïve

388   conditions in this study. A 1mL blunt syringe was used to infiltrate the leaf from

389   the abaxial side, through holes poked with a needle per half of the leaf. 24 leaves

390   per timepoint per treatment were used. Each experiment was repeated 3 times

391   and each produced a sequencing data set.

392   18-22 hours after the treatment, *Pto* was syringe-infiltrated into the treated leaves.

393   To prepare the inoculum, *Pto* strains were first spread on King's B Agar (King et

394   al. 1954) supplemented with 20µg/mL rifampicin and grown to lawns overnight at

395   room temperature. Cells from the lawn were harvested and resuspended in

396   0.25mM MgCl2 to an optical density 600nm of 0.8. The resuspension was used

397   as the inoculum. After infiltration, excess liquid on the leaves were soaked up

398   with paper towels. The bacteria strains used are listed in Table 4.

399 At 5 hpi, leaves were cut at the petiole-leaf blade junction. The leaves were then

400 lined up in the middle of a sheet of parafilm. The parafilm was then folded so that

401 the leaves are held between two layers of the parafilm with the cut side pointing

402 at the folding line. Small openings were cut at the folding line before the

403 assembly was rolled up from side to side and inserted into the barrel of 20-mL

404 syringes. The syringes were put in 50mL centrifuge tubes and an RNA stabilizing

405 buffer (Wit et al. 2012) was poured into the syringe. The tubes were then

406 vacuumed at 96kPA for 2 min followed by a slow release. The vacuum procedure

407 was repeated twice to infiltrate the leaves with the buffer. The excess buffer was

408 discarded, then the tubes with syringes inside were centrifuged at 1,000x g for 10

409 minutes at 4°C to collect the apoplast wash fluid. The collected fluid was then

410 passed through 0.20-µm Micropore Express Plus membrane filters (Millipore) to

411 collect the bacteria. The filters were then placed in homogenization tubes and

412 frozen by liquid nitrogen before storing at –80°C.

413 The filters were homogenized using Geno/Grinder (SPEX SamplePrep) for 1 min

414 at 1,750 Hz on a liquid nitrogen chilled sample holder. Trizol (Thermo Fisher

415 Scientific) was then added, and the Direct-Zol RNA Miniprep Plus Kit (Zymo

416 Research) or Monarch RNA isolation kit (NEB) was used for RNA extraction. An

417 additional TURBO DNase treatment (Ambion, Invitrogen/Thermo Fisher) was

418 carried out to further reduce DNA content, followed by a cleanup step using

419 Monarch RNA cleanup kit (NEB). Finally, the library was produced using the

420 TruSeq Stranded Total RNA library prep kit (Illumina). RNA sample was then

421 sent to Georgia Genomics and Bioinformatics Core for quantification, QC

422 analysis and RNA sequencing. Single-end 75nt reads were sequenced using

423 Nextseq 500 system (Illumina) in high output mode.

424

425 Data analysis

426 The sequencing reads were first trimmed using Trimmomatic V0.36 and read

427 counts were computed using EDGE-pro V1.3.1 and exported using

428 edgeToDeseq.perl. Differentially-expressed genes were then identified using

429 DESeq2 V1.28.1, with an adjusted P value below 0.05 and a log fold change

430 over 0.58. Venn diagram was generated with InteractiVenn website (Heberle et al.

431 2015) and meta-chart website. Illumina sequencing data were deposited in the

432 Gene Expression Omnibus under accession number GSE191032.

433 Protein function groups were manually curated by combining the outcome of

434 Kyoto Encyclopedia of Genes and Genomes (KEGG), NCBI protein, Uniprot,

435 pseudomonas.com, and literature mining. Protein cytoplasm or membrane

436 localization was determined by checking pseudomonas.com and Uniprot.

437

438 RT-qPCR

439 For real time quantification PCR analysis of selected genes, plants were treated

440 in the same way as described above. At 5 hpi, three 0.4mm diameter leaf disks

441 from inoculated leaves were taken and frozen by liquid nitrogen. Leaves were

442 then crushed by Geno/Grinder in liquid nitrogen chilled sample holder. Then

443 Trizol was added before RNA was extracted using Monarch RNA Cleanup Kit

444 (NEB), skipping the gDNA cleaning column steps. After the extraction, the

445 sample was treated with TURBO DNase. Monarch total RNA miniprep kit was

446 then used to clean up the reaction. The RNA was then reverse-transcribed using

447 QuantaBio qScript cDNA SuperMix. Luna qPCR Master Mix (NEB) was used for

448 reaction setup, and StepOnePlus (Applied Biosystems) was used to carry out the

449 PCR reaction. Reference genes (*hemD, Isc-1,* and 16s rRNA) were chosen from

450 the list published previously (Smith et al. 2018), and showed minimal change in

451 expression between conditions, with expression level close to genes of interest,

452 based on the RNA-seq data. Raw data from StepOnePlus was then analyzed

453 using LinRegPCR, and expression fold change was calculated using Microsoft

454 Excel. Primers used are listed in table 5.

455 **Acknowledgements**

460 Table 1. Function groups of AlgU-induced genes in naïve leaves compared to King's B media.

Number of genes	Function
36	Hypothetical and unknown function
26	Transporter, permease, and lipoprotein
23	Amino acid, nucleotide, lipid metabolism, and energy production
19	T3SS related
19	Transcription regulator
16	tRNA

14	Protein translation and folding
13	Alginate synthesis
10	Ribosomal protein
9	Osmotic stress
6	T2SS structural
6	Oxidative response
5	Cell shape maintenance and division
5	DNA repair
4	Coronatine synthesis
3	Phage related
2	Iron-sulfur cluster related
2	Other secretion system
2	SOS response
2	Antibiotic resistance and toxin/antitoxin
2	Plasmid mobility gene

461

462

463

464   Table 2. Function groups of AlgU down-regulated genes in naïve leaves.

Number of genes	Function
35	Hypothetical and unknown function
32	Amino acid, nucleotide, and lipid metabolism, and energy production
14	Transporter, permease, and lipoprotein
13	Transcription regulator
11	Flagella related
10	Chemotaxis
10	Receptor and signal transduction
8	Conjugal transfer protein
6	Protein translation, folding, and protease
5	Phage or transposase
3	GGDEF domain containing protein
3	DNA modification
3	Sulfur metablism or iron-sulfur cluster related
1	Antibiotic synthesis

465

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468

469   Table 3. PTI intervenes almost all AlgU-regulated function groups.

470   AlgU-induced genes:

Percent of *in planta* regulon[a]	Number of genes	Function
61.54%	16	Transporter, permease, and lipoprotein
100.00%	13	Alginate synthesis
36.11%	13	Hypothetical and unknown function
52.17%	12	Amino acid, nucleotide, lipid metabolism, and energy production
57.89%	11	T3SS related
78.57%	11	Protein translation and folding
90.00%	9	Ribosomal protein
47.37%	9	Transcription regulator
88.89%	8	Osmotic stress
100.00%	4	Coronatine synthesis
50.00%	3	T2SS structural
100.00%	3	Phage related
40.00%	2	Cell shape maintenance and division
12.50%	2	tRNA
100.00%	2	Other secretion system
40.00%	2	DNA repair
33.33%	2	Oxidative response
50.00%	1	Antibiotic resistance and toxin/antitoxin
50.00%	1	Plasmid mobility gene
0.00%	0	Iron-sulfur cluster related
0.00%	0	SOS response

471   AlgU down-regulated genes:

Percent of *in planta* regulon	Number of genes	Function
53.13%	17	Amino acid, nucleotide, and lipid metabolism, and energy production
45.71%	16	Hypothetical and unknown function
90.91%	10	Flagella related
71.43%	10	Transporter, permease, and lipoprotein
76.92%	10	Transcription regulator
90.00%	9	Chemotaxis
70.00%	7	Receptor and signal transduction
50.00%	3	Protein translation, folding, and protease
66.67%	2	GGDEF domain containing protein
40.00%	2	Phage or transposase
66.67%	2	Sulfur metabolism or iron-sulfur cluster related
100.00%	1	Antibiotic synthesis

| 12.50% | 1 | Conjugal transfer protein |
| 33.33% | 1 | DNA modification |

a. Percent of genes in each function groups in Table 1 or 2 that are intervened by PTI.

Table 4. Bacteria strains used.

Stock #	Genotype	Plasmid	Reference
GS_00950	*Pto* DC3000 Wild type	pJN105	This study
GS_00585	*Pto* DC3000 *ΔalgUmucAB*	pJN105	Markel et al. 2016

Table 5. Primers used for RT-qPCR.

hemD, *lsc-1*, and 16s primers were used in Smith et al. 2018 and were used as reference genes in this study. Primer sequences of *algD* is from Markel et al. 2016. Gene IDs are based on GenBank *Pto* genome NC_004578.1.

Gene ID	Name	Forward primer	Reverse primer
PSPTO_0129	*hemD*	TCAGCAGCAGTCTGCCTTTA	GTTGCTGAACCCACACTGAA
PSPTO_1453	*lsc-1*	TCTTTGGTGGTCGTGTAATGG	GGTGTGACGCAGGTGTAATAA
multiple	*16s*	ACGGGTACTTGTACCTGGTG	CGTTTCCGAGCGTTATCCC
PSPTO_4575	*opuCA*	AACCGTCTGATCATGCCGAC	TGGATCACGTAGCCGATGTTG
PSPTO_1951	*fliD*	TGCAGGGCAAAGGCATTACC	TCGTCGAACTGAAGACCAGC
PSPTO_4001	*avrPto1*	TCCAGTCAACTGCTGAGCG	TCAGGCTTTGAGGTGCTTGG
PSPTO_1243	*algD*	GAAGAACGGCGACCTGGAACTG	CGGTGCTGCGAACCACGATAG

Figure Legends.

Figure 1. Venn diagram showing numbers of genes differentially-regulated under different conditions. **A**. AlgU *in planta* regulon genes is present in both left(circle): upregulated (top) or downregulated (bottom) in wild type compared to *ΔalgU Pto*, and right(grey): upregulated (top) or downregulated (bottom) in naïve leaves compared to King's B growth media. **B**. Proportion of genes upregulated (top) or downregulated (bottom) by AlgU in plant (circle) overlaps with genes that are

492   expressed more in mock-treated leaves (naïve) compared to flg22-treated (PTI

493   pre-induced) leaves (grey).

494

495   Figure 2. Expression changes of stress response related genes. **A**. Osmotic

496   stress response genes. **B**. Alginate synthesis genes. * indicates genes with

497   padj >0.05 calculated by DESeq2. All * in this graph are from grey bars. Arrows

498   indicate genes within an operon.

499

500   Figure 3. Expression changes of Type 3 Effectors and Type 3 Secretion System

501   (T3SS) related genes that were identified as AlgU regulated in this study. *

502   indicates genes with padj >0.05 calculated by DESeq2. All * in this graph are

503   from grey bars. Arrows indicate genes within an operon.

504

505   Figure 4.

506   Figure 4. Expression changes of Type 2 Secretion System (T2SS) pathway

507   genes. * indicates genes with padj >0.05 calculated by DESeq2. All * in this

508   graph are from grey bars. Arrows indicate genes within an operon.

509   Figure 5. Expression changes of motility genes. **A**. Sketch showing the four

510   classes belong to the motility gene regulatory hierarchy. **B**. Log2 fold change of

511   the motility genes organized by classes. **C**. Genes related to swarming motility

512   (syringafactin) and flagella glycosylation. * indicates genes with padj >0.05

513   calculated by DESeq2. * for black bars are placed under line of 0, * for grey bars

514   are placed above line of 0. Arrows indicate genes within an operon.

515   Figure 6. Expression changes of transcriptional regulators that are differentially-

516   expressed in the absence of AlgU in naïve plant. **A**. Genes downregulated in

517   *ΔalgU* background. **B**. Genes upregulated in *ΔalgU* background. * indicates

518   genes with padj >0.05 calculated by DESeq2. All * in this graph are from grey

519   bars.

520

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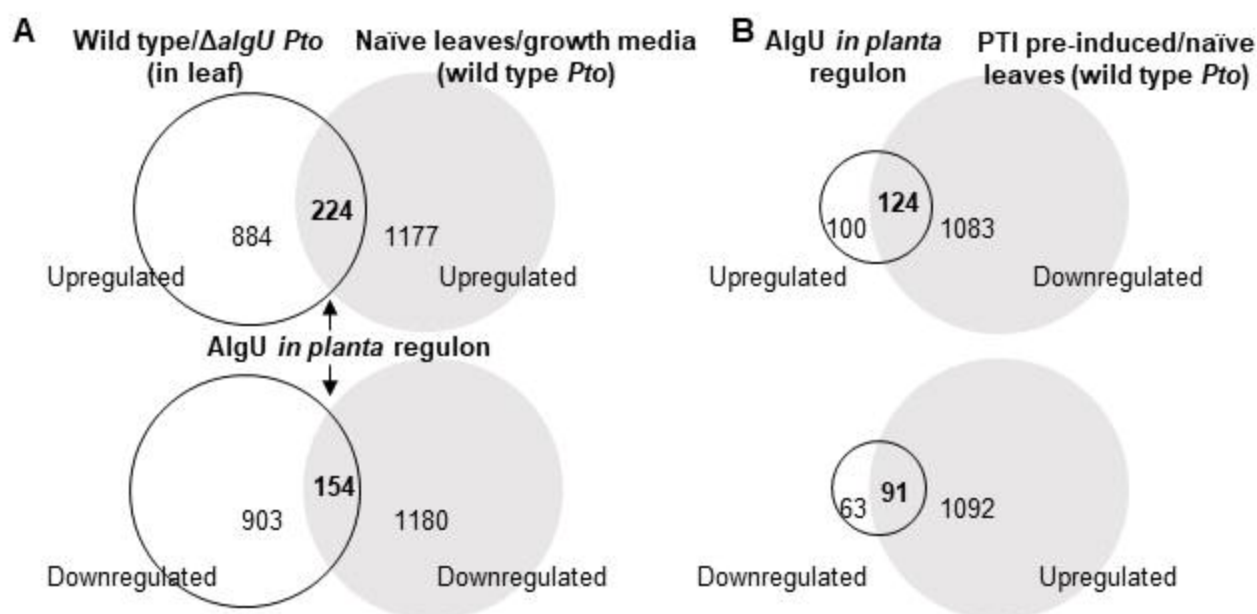700   428:764–767
701

Figure 1

A

Wild type/ΔalgU Pto (in leaf)	Naïve leaves/growth media (wild type Pto)

224

884   1177

Upregulated   Upregulated

AlgU *in planta* regulon

154

903   1180

Downregulated   Downregulated

B

AlgU *in planta* regulon	PTI pre-induced/naïve leaves (wild type Pto)

124

100   1083

Upregulated   Downregulated

91

63   1092

Downregulated   Upregulated

Figure 1. Venn diagram showing numbers of genes differentially-regulated under different conditions. **A**. AlgU *in planta* regulon genes is present in both left(circle): upregulated (top) or downregulated (bottom) in wild type compared to *ΔalgU Pto*, and right(grey): upregulated (top) or downregulated (bottom) in naïve leaves compared to King's B growth media. **B**. Proportion of genes upregulated (top) or downregulated (bottom) by AlgU in plant (circle) overlaps with genes that are expressed more in mock-treated leaves (naïve) compared to flg22-treated (PTI pre-induced) leaves (grey).
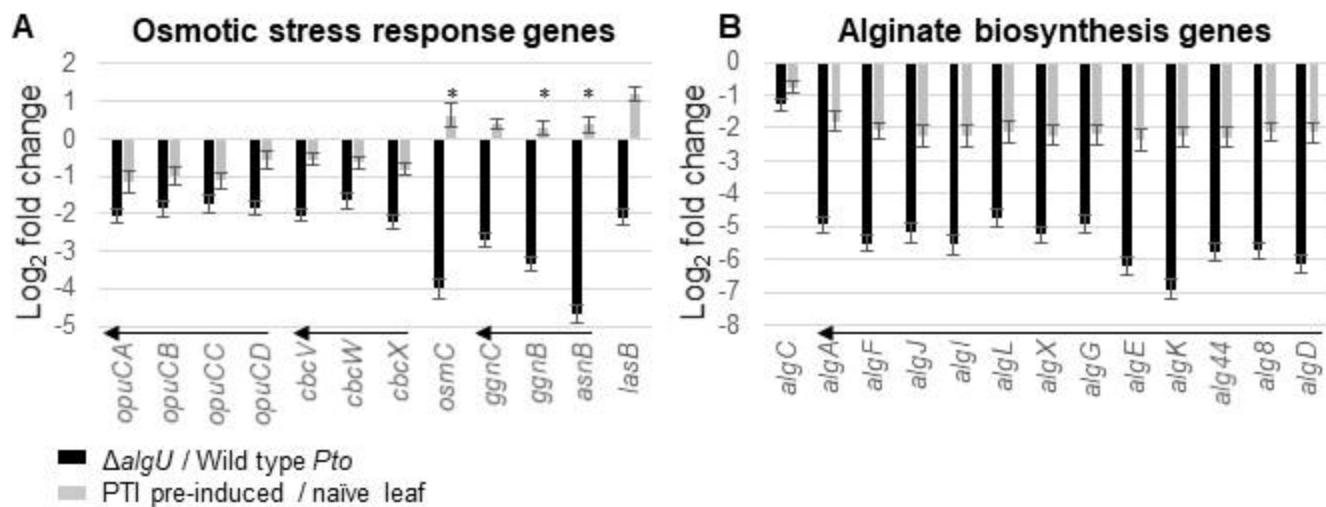
Figure 2

Figure 2. Expression changes of stress response related genes. **A.** Osmotic stress response genes. **B.** Alginate synthesis genes. * indicates genes with padj >0.05 calculated by DESeq2. All * in this graph are from grey bars. Arrows indicate genes within an operon.
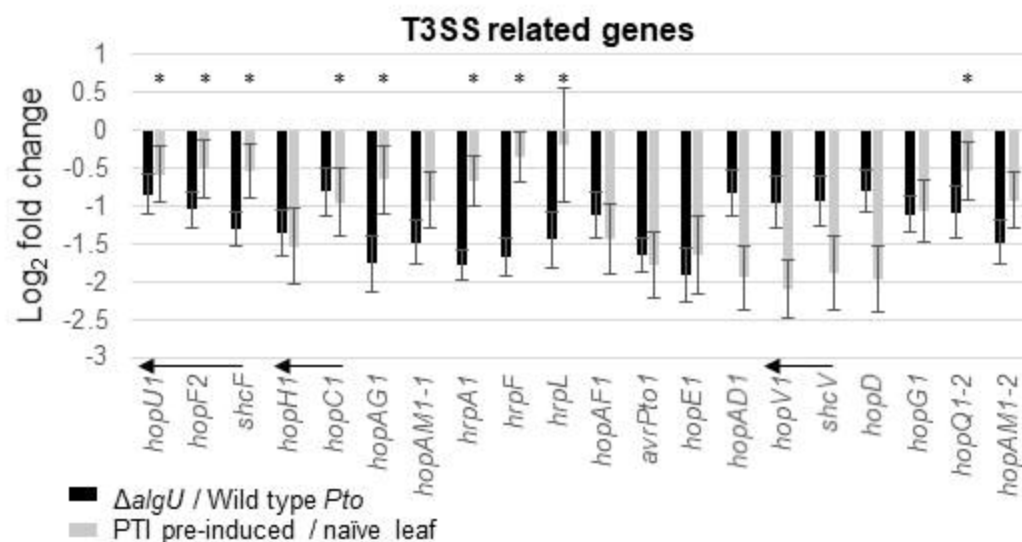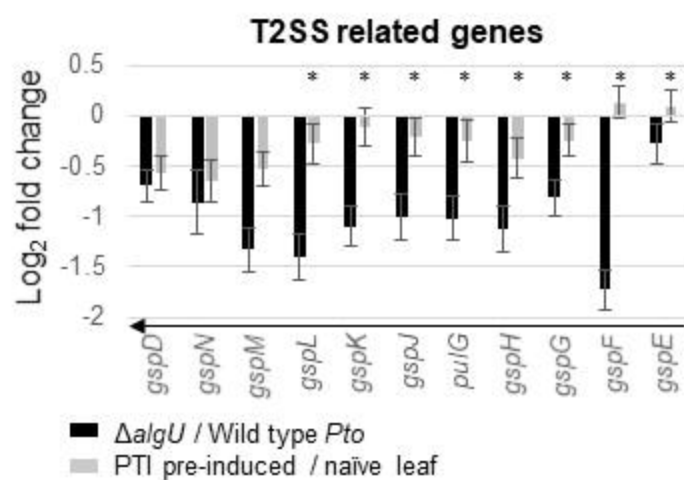
Figure 3

Figure 3. Expression changes of Type 3 Effectors and Type 3 Secretion System (T3SS) related genes that were identified as AlgU regulated in this study. * indicates genes with padj >0.05 calculated by DESeq2. All * in this graph are from grey bars. Arrows indicate genes within an operon.

Figure 4

Figure 4. Expression changes of Type 2 Secretion System (T2SS) pathway genes. * indicates genes with padj >0.05 calculated by DESeq2. All * in this graph are from grey bars. Arrows indicate genes within an operon.
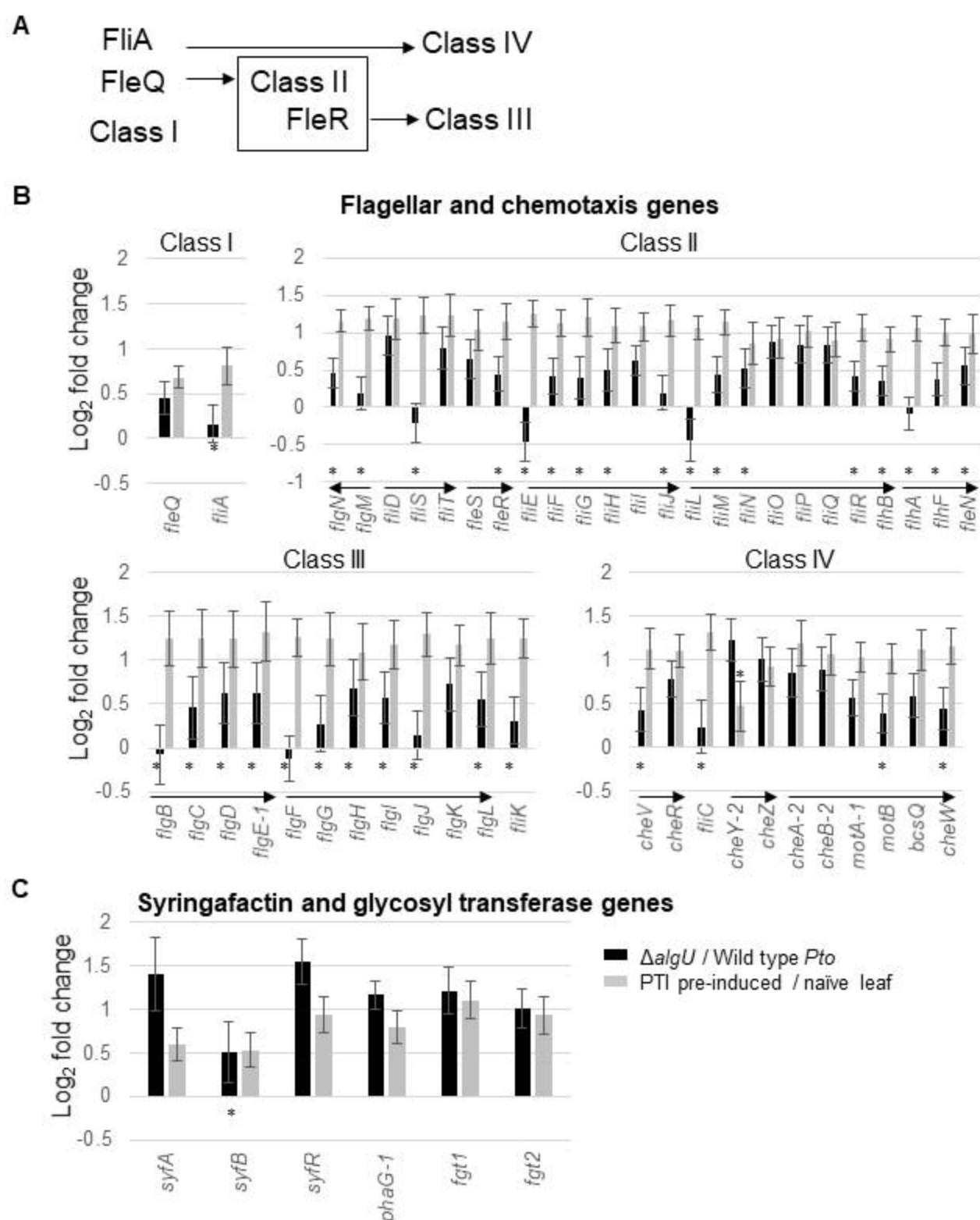
Figure 5

Figure 5. Expression changes of motility genes. **A.** Sketch showing the four classes belong to the motility gene regulatory hierarchy. **B.** Log2 fold change of the motility genes organized by classes. **C.** Genes related to swarming motility (syringafactin) and flagella glycosylation. * indicates genes with padj >0.05 calculated by DESeq2. * for black bars are placed under line of 0, * for grey bars are placed above line of 0. Arrows indicate genes within an operon.
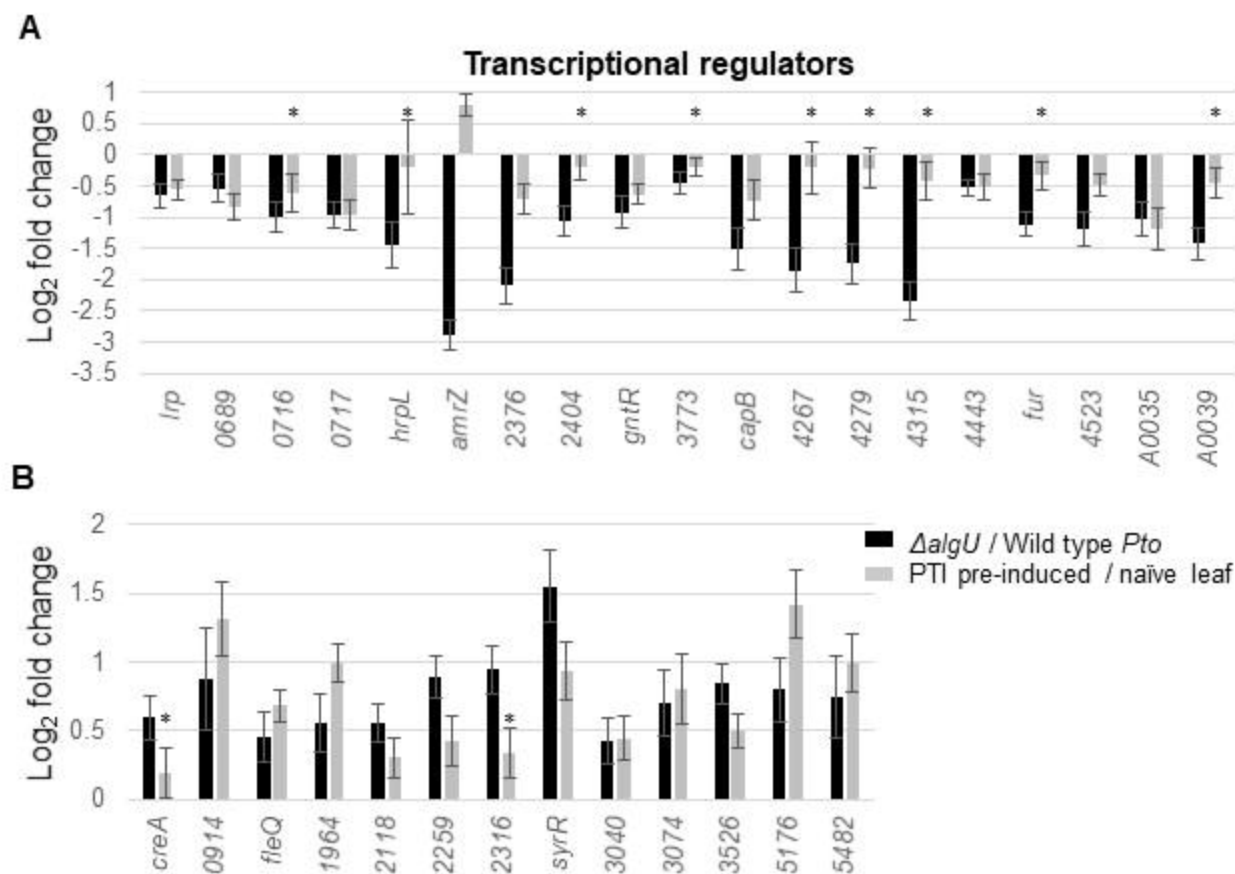
Figure 6

A

B

Figure 6. Expression changes of transcriptional regulators that are differentially-expressed in the absence of AlgU in naïve plant. **A**. Genes downregulated in *ΔalgU* background. **B**. Genes upregulated in *ΔalgU* background. * indicates genes with padj >0.05 calculated by DESeq2. All * in this graph are from grey bars.