

1 **Architecture of genome-wide transcriptional regulatory network reveals**
2 **dynamic functions and evolutionary trajectories in *Pseudomonas syringae***

3 Yue Sun^{#1}, Jingwei Li^{#1}, Jiadai Huang^{#1}, Shumin Li², Youyue Li¹, Xin Deng^{*1,3,4,5}

4

5 ¹ Department of Biomedical Sciences, City University of Hong Kong, Hong Kong,
6 China

7 ² The University of Hong Kong, Pokfulam, Hong Kong, China

8 ³ Shenzhen Research Institute, City University of Hong Kong, Shenzhen, Guangdong,
9 China

10 ⁴ Tung Biomedical Sciences Center, City University of Hong Kong, Hong Kong,
11 China

12 ⁵ Chengdu Research Institute, City University of Hong Kong, Chengdu, China

13 *To whom correspondence should be addressed: Dr. Xin Deng. Tel: +852 3442 5693;

14 Fax: +852 3442 0549; E-mail: xindeng@cityu.edu.hk.

15 # These authors contributed equally to the paper as the first authors.

16 **Abstract**

17 The model Gram-negative plant pathogen *Pseudomonas syringae* utilises hundreds of
18 transcription factors (TFs) to regulate its functional processes, including virulence and
19 metabolic pathways that control its ability to infect host plants. Although the
20 molecular mechanisms of regulators have been studied for decades, a comprehensive
21 understanding of genome-wide TFs in *P. syringae* remains limited. Here, we
22 investigated the binding characteristics of 170 of 301 annotated TFs through ChIP-seq.
23 Fifty-four TFs, 62 TFs and 147 TFs were identified in top-level, middle-level and
24 bottom-level, reflecting multiple higher-order network structures and direction of
25 information-flow. More than forty thousand TF-pairs were classified into 13
26 three-node submodules which revealed the regulatory diversity of TFs in *P. syringae*
27 regulatory network. We found that bottom-level TFs performed high co-associated
28 scores to their target genes. Functional categories of TFs at three levels encompassed
29 various regulatory pathways. Three and 25 master TFs were identified to involve in
30 virulence and metabolic regulation, respectively. Evolutionary analysis and
31 topological modularity network revealed functional variability and various
32 conservation of TFs in *P. syringae*. Overall, our findings demonstrated the global
33 transcriptional regulatory network of genome-wide TFs in *P. syringae*. This
34 knowledge can advance the development of effective treatment and prevention
35 strategies for related infectious diseases.

36 **Keywords:** transcription factor; *Pseudomonas syringae*; regulatory network

37 **Introduction**

38 Transcription is a pivotal process in cellular life events. Transcription factors (TFs)
39 play a crucial role in this process by acting as key regulators that coordinate various
40 biological activities(Lee & Young, 2013; Papavassiliou & Papavassiliou, 2016). TFs
41 control the recruitment of RNA polymerase by identifying and binding to the
42 promoters of downstream genes, thereby either activating or repressing the expression
43 of target genes(Lambert et al., 2018; Wade, 2015). Numerous studies have focused on
44 regulatory events in eukaryotic species, such as humans(Jolma et al., 2013),
45 mice(Badis et al., 2009), and *Saccharomyces cerevisiae*(C. Zhu et al., 2009), and
46 prokaryotic species, such as *Escherichia coli*(Shen-Orr, Milo, Mangan, & Alon, 2002).
47 However, limited comprehensive TF binding datasets for microbial pathogens are
48 available.

49 *Pseudomonas syringae*, an important Gram-negative phytopathogen and a model
50 pathogenic bacterium, infects many plants, including economically valuable crops,
51 resulting in substantial annual economic losses globally(Hirano & Upper, 2000).
52 Upon entering host cells, *P. syringae* employs several strategies, such as changing its
53 motility type and secreting phytotoxins, to overcome the plant's immune defences and
54 establish colonies(Bender, Alarcón-Chaidez, & Gross, 1999; Ichinose, Taguchi, &
55 Mukaihara, 2013; Taguchi & Ichinose, 2011). *P. syringae* causes severe disease by
56 secreting various effector proteins through the needle-like type III secretion system
57 (T3SS); this process is regulated by a cluster of TFs(Cunnac, Lindeberg, & Collmer,
58 2009; Hendrickson, Guevera, & Ausubel, 2000; Huang, Yao, Sun, Ji, & Deng, 2022;

59 Jingru Wang et al., 2018). The alternative sigma factor RpoN activates the
60 transcription of another alternative sigma factor, HrpL, which, in turn, binds to the
61 pathogenicity (*hrp*) box in the promoter region of T3SS genes, regulating most of
62 these T3SS genes(Alfano & Collmer, 1997; Lan, Deng, Zhou, & Tang, 2006;
63 Yingxian Xiao & Hutcheson, 1994). HrpS is one of the most important TFs that
64 regulate numerous biological processes(Jingru Wang et al., 2018). Its heterodimeric
65 complex, HrpRS, is modulated by at least six two-component systems (TCSs):
66 RhpRS(Deng et al., 2014), CvsRS(Fishman, Zhang, Bronstein, Stodghill, & Filiatrault,
67 2018), GacAS(Chatterjee et al., 2003), AauRS(Yan, Rogan, Pang, Davis, & Anderson,
68 2020), CbrAB2 and EnvZ-OmpR(Shao et al., 2021). In particular, RhpRS serves as a
69 master regulator of T3SS in *P. syringae*. RhpRS senses plant-derived signals, such as
70 polyphenols, through the histidine kinase Pro40 within RhpS and controls the
71 expression of T3SS genes in response to environmental stress(Deng et al., 2014;
72 Yanmei Xiao et al., 2007; Xie et al., 2021). Within the sensor region, the cognate
73 response regulator RhpR undergoes modulation in its phosphorylation state by RhpS,
74 thereby regulating a group of T3SS genes(Deng et al., 2010). Phosphorylated RhpR
75 directly binds to the *hrpRS* promoter, suppressing the *hrpRS* operon and the
76 subsequent *hrpRS-hrpL-hrp* cascade(Deng et al., 2010; Deng et al., 2014; Deng, Xiao,
77 Lan, Zhou, & Tang, 2009; Shao, Xie, Zhang, & Deng, 2019; Yanmei Xiao et al.,
78 2007).

79 Recently, through a combined analysis of RNA sequencing (RNA-seq) and chromatin
80 immunoprecipitation sequencing (ChIP-seq), we identified seven additional TCSs

81 (ErcS, Dcsbis, PhoBR, CzcSR, AlgB/KinB, MerS and CopRS) that regulate the
82 virulence of *P. syringae*(Xie et al., 2022). In addition, we developed an intricate
83 PSTCSome (*P. syringae* TCS regulome) network containing numerous functional
84 genes that respond to changing environmental conditions(Xie et al., 2022).
85 Furthermore, we examined the overall crosstalk between 16 virulence-related
86 regulators under different growth conditions, such as King's B and minimal media. By
87 analysing differentially expressed genes and binding peaks, we constructed a *P.*
88 *syringae* regulatory network (PSRnet), revealing the involvement of hundreds of
89 functional genes in virulence pathways(Shao et al., 2021). We also elucidated the
90 molecular mechanisms and functions of TFs binding within coding sequences (CDS)
91 and found that CDS-binding TFs interact with cryptic promoters in coding regions,
92 thereby regulating the expression of subgenus and antisense RNAs(Hua et al., 2022).
93 We propose a luminescence reporter system designed to quantitatively measure the
94 translational elongation rates (ERs) of T3SS-related proteins. Our findings
95 demonstrate the key roles of transfer RNAs (tRNAs) and elongation factors in
96 modulating translational ERs and facilitating T3SS protein synthesis(Sun et al., 2022).
97 Although many key virulence regulators in *P. syringae* have been studied, the global
98 regulatory mechanism and interactions of all 301 annotated TFs across various
99 biological processes remain unclear. To comprehensively explore the DNA-binding
100 features and map the transcriptional regulatory network of all TFs in *P. syringae*, we
101 constructed 170 TF-overexpressing strains and used ChIP-seq, a highly effective and
102 important technology for analysing protein–DNA interactions(Mathelier, Shi, &

103 Wasserman, 2015). This analysis not only provided insights into the interactions
104 between TFs and their target genes but also revealed the hierarchy (top, middle and
105 bottom) and co-association scores of all these TFs. We found that more than half of
106 270 TFs in downstream position tended to be regulated by top TFs and bound to the
107 target genes with high co-associated scores. Different TF-pairs were classified into 13
108 basic three-node submodules, including ringent loops and locked loops. In addition,
109 we mapped the hierarchical binding network of TFs and identified three
110 virulence-related master TFs and 23 metabolic master TFs. Furthermore, we
111 employed ChIP-seq to determine the binding sites of 5 TFs in 4 *P. syringae* lineages,
112 revealing the diversity of TF binding events and the varying functions of TFs among
113 different *P. syringae* strains. Topological modularity classification of the network,
114 including TFs and target genes, revealed the diverse biological functions of TFs in *P.*
115 *syringae*. This study provides a global and convenient platform for understanding the
116 transcriptional regulatory characteristics and biological functions of TFs in *P. syringae*.
117 In addition, this study provides valuable insights that can inform the development of
118 effective therapies for not only *P. syringae* but also other associated infectious
119 diseases.

120 **Results**

121 **ChIP-seq analysis revealed the binding specificities of 170 previously**
122 **uncharacterised TFs in *P. syringae***

123 Based on the current annotations available on ‘*Pseudomonas* Genome DB’
124 (<https://www.pseudomonas.com/>)(Winsor et al., 2016), we initially determined the
125 locations of all 301 annotated TFs in the *P. syringae* genome (**Figure S1a**). To
126 elucidate the binding preferences and functional characteristics of TFs of *P. syringae*,
127 we performed ChIP-seq for the 170 TFs, including three (1.8%) predicted
128 transcriptional regulators, 132 (77.6%) annotated transcriptional regulators and 35
129 (20.6%) functional proteins with DNA-binding annotations. Based on the
130 DNA-binding domains as annotated in the transcription factor prediction
131 database(Wilson, Charoensawan, Kummerfeld, & Teichmann, 2008), we categorised
132 the 170 analysed TFs into 25 families(Fan et al., 2020). The majority of TFs belonged
133 to the LysR, TetR, AsnC, GntR and AraC families. Among these TFs, PSPPH4700,
134 PSPPH3798, CysB, PSPPH1951, PSPPH4638, PSPPH3504, PSPPH3268 and Irp
135 exhibited over 1,000 binding peaks (**Figure S1b**). The enriched loci of these binding
136 peaks indicated that these TFs displayed a significant preference for binding to
137 promoters, directly regulating the transcription of downstream targets (**Figure S1c**).
138 The peak loci of 10 TFs (PSPPH0286, PSPPH0411, PSPPH0711, PSPPH1734,
139 PSPPH2357, PSPPH2407, PSPPH2862, PSPPH3155, PSPPH3431, PSPPH3468,
140 PSPPH4127, PSPPH4622 and PSPPH4768) were completely enriched in the
141 promoter region, with the majority of them belonging to the LysR family. Taken

142 together, the 170 tested TFs in *P. syringae* had over 26,000 DNA-binding peaks
143 distributed across different regions of target genes, suggesting their direct regulatory
144 functions.

145 **Hierarchical TFs reflected multiple higher-order network structures**

146 Transcriptional changes in bacteria are often manipulated by a complex network of
147 TFs. However, bacterial TFs usually have been studied individually or in small
148 clusters with related functions. To comprehensively investigate the associations of all
149 TFs in *P. syringae* at a system level, we constructed a hierarchical network of 270
150 analysed TFs. The findings revealed 1,757 TF interactions among these 26,000
151 binding events (**Supplementary Table 1a**). Subsequently, we computed information
152 flow parameters for each TF (Gerstein et al., 2012). In brief, we defined out-degree (O)
153 and in-degree (I) as the number of interactions of a TF in the hierarchical network,
154 representing the regulation of other factors by this TF and the regulation of this TF by
155 other factors, respectively. The difference between O and I indicated the direction of
156 information flow in the network. Hierarchy height (H) was defined as the normalised
157 metric of information circulation, calculated as $H = (O - I) / (O + I)$. When H was close
158 to 1 ($H \approx 1$), it indicated that these TFs tended to regulate other factors and occupy
159 upstream positions in the network. Conversely, when H was close to -1 ($H \approx -1$), it
160 indicated that these TFs were more likely to be regulated than to regulate other TFs,
161 occupying downstream positions in the network. Based on these criteria, we
162 categorised the 270 analysed TFs into three levels: 54 (20%) executive TFs (such as
163 AlgQ, LexA2 and PSPPH0222) at the top level, 62 (23%) communicative TFs (such

164 as MexT, PsrA and PSPPH1100) at the middle level and 147 (54%) foreman TFs
165 (such as PobR, DksA2 and PSPPH0755) at the bottom level (**Figure 1a**,
166 **Supplementary Table 1b**). The presence of a larger number of TFs (147) at the
167 bottom level indicated a high degree of information flow, suggesting the maximisation
168 of the number of upward-pointing edges in the network.

169 The hierarchical network revealed a downward information flow, suggesting the
170 prioritisation of collaboration between different hierarchy levels. Therefore, we
171 defined the direct binding between two TFs as a direct interaction and investigated
172 collaborations within and between hierarchy levels, specifically intra-level (TT, MM
173 and BB) and inter-level (TM, TB and MB) interactions (**Figure 1b, Supplementary**
174 **Table 1a**). In terms of the top-level TFs, physical interactions became more enriched
175 as the hierarchy level of their collaborators decreased. Direct interactions between TB
176 pairs constituted the most substantial portion, accounting for nearly half of all
177 interactions. A similar pattern was observed among the bottom-level TFs, where
178 interactions diminished as the hierarchy level of their collaborators decreased.
179 Compared with interactions among the top- and bottom-level TFs, middle-level TFs,
180 serving as information transmission centres, exhibited lower levels of intra-level
181 collaborations. In summary, transcriptional regulation within the TF hierarchy was
182 predominantly manipulated by top-level TFs, which directed the flow of information
183 to downstream TFs.

184 **Multiple three-node submodules revealed the regulatory diversity of TFs in the *P.***
185 ***syringae* regulatory network**

186 Natural networks, including transcriptional regulation networks, usually show
187 complex characteristics(Newman, 2001; Strogatz, 2001). Among complex networks,
188 some small-scale networks demonstrate numerous connections between individual
189 information nodes and information clusters(Amaral, Scala, Barthelemy, & Stanley,
190 2000; Jeong, Tombor, Albert, Oltvai, & Barabási, 2000). To investigate the basic
191 structural features of our transcription network, we defined directed edges as direct
192 interactions between two TF nodes and identified global submodules comprising
193 different TF nodes. In this study, we specifically focused on three-node modules,
194 which were considered as ‘network motifs’(Milo et al., 2002). Using algorithms
195 designed to detect recurring modules(Shen-Orr et al., 2002), we scanned our hierarchy
196 network and identified 40,307 different pairs across 13 basic three-node submodules
197 **(Figure 1c)**.

198 In the first six submodules, we observed that two TF nodes established a relationship
199 only through another node. We denoted these submodules (M1 to M6) as ‘ringent
200 loops’. These seemingly simpler regulation modules appeared more in the *P. syringae*
201 transcriptional regulatory network, especially the first module (M1, n = 24,479),
202 indicating that *P. syringae* favours the use of simple but efficient modes for
203 modulating transcriptional regulation. For example, PhnF and FruR were directly
204 regulated by CysB (M1), and CapA was coregulated by Anr and PSPPH4700
205 simultaneously (M3, n = 6,177). In addition, M6 (n = 20) contained pairs of mutually
206 regulating TFs (toggle switches), such as PSPPH3268, PSPPH2315 and PSPPH2733.

207 The remaining seven submodules, denoted as ‘locked loops’ (M7 to M13), comprised

208 subordinate three-node regulatory modules within our network. Notably, no instances
209 of a ‘fully connected triad’ (M12) were observed in our network ($n = 0$). We found 50
210 ‘self-loop’ submodules (M7) in our network, including CspD, PSPPH4694 and
211 PSPPH3798, which engaged in mutual regulatory interactions. Among these
212 submodules, M9 ($n = 10$) was the least common and contained six pairs of toggle
213 switches involving mutually regulating TFs (such as PSPPH1951, PSPPH4612 and
214 CysB), which were similar to those found in the human transcriptional regulatory
215 network(Gerstein et al., 2012). Notably, the most enriched locked loop in our network
216 was M13 ($n = 696$), denoted as a ‘feed-forward loop’, which has been extensively
217 studied in other species such as humans and *E. coli*. In this submodule, upstream TFs
218 regulated targeted TFs either by binding directly or manipulating other TFs (**Figure**
219 **S2**). For example, TF Irp directly controlled PSPPH4899 and also indirectly regulated
220 it by binding to Lon.

221 Taken together, the simplest and most effective submodule M1 and the coregulatory
222 submodule M13 played crucial roles in the transcriptional regulation of TFs in *P.*
223 *syringae*. In addition, we found 92 auto-regulators in our hierarchy network. These
224 auto-regulators are important and always act as repressors in scenarios of
225 multi-stability, such as in plant intercellular spaces where bacteria grow (**Figure**
226 **1d**)(Alon, 2007). These regulators are regarded as bistable switches that further
227 influence the expression of downstream genes(Burda, Krzywicki, Martin, & Zagorski,
228 2011). For example, our previous study demonstrated that Lon is a dual-function
229 regulator involved in the regulation of virulence and metabolism in *P. syringae*(Hua et

230 al., 2020). Lon was identified as an auto-regulator in this study. Furthermore, DksA2,
231 which is widely regarded as a protective protein against oxidative stress(Fortuna et al.,
232 2022), was identified as a new bistable switch in this study. In summary, the
233 classification of the TF hierarchy and the identification of enriched network modules
234 not only offer functional predictions for transcriptional regulators but also provide
235 insights into the communication network that governs TF regulation in *P. syringae*.

236 **High co-association pairs occurred more in bottom-level TFs in *P. syringae***

237 In addition to direct interactions between TFs, we found a notable preference for
238 co-binding peaks among different TF pairs. Briefly, we counted overlapping regions
239 within the binding peaks of all TF pairs. The ratio of intersection regions to the union
240 set of all peaks between two TFs was identified as the genome-wide co-association of
241 specific TFs. We first computed co-association scores between TF pairs across three
242 hierarchy levels. The majority of TFs tended to cooperate with other TFs and co-bind
243 to specific genomic regions (**Figure S3a, c and e**). To identify the potential functions
244 of TFs in each level, we performed functional annotations using hypergeometric tests
245 (BH-adjusted $p < 0.05$) based on gene sets derived from the Gene Ontology (GO) and
246 Kyoto Encyclopaedia of Genes and Genomes (KEGG) databases (**Figure S3b, d and**
247 **f, Supplementary Table 2a, c–d**). The functional categories of TFs at these three
248 levels encompassed various regulatory pathways. For example, the top-level TF
249 PSPPH4700 was involved in siderophore transportation and phosphorelay signal
250 transmembrane transportation. The middle-level TF Lon regulated GTP binding and
251 ribosome transcription. The bottom-level TF PSPPH3486 was involved in amino acid

252 transportation, PSPPH0101 in ATP binding and PSPPH1049 in catalytic activity.

253 Notably, we found that TFs at the top level, without cooperating TFs, exhibited a large

254 number of binding peaks (**Figure S3a**). This finding suggested that these TFs

255 preferred to regulate target genes by recruiting to specific sites of other TFs,

256 facilitating the direct binding between other TFs and their specific targets, a

257 phenomenon defined as tethered binding(Jie Wang et al., 2012). For example, the

258 top-level TF PSPPH4700 (yielding over 1,700 peaks) cooperated with only 24

259 executive TFs, resulting in an average co-association score of 0.05 (**Supplementary**

260 **Table 2b**). PSPPH4700 directly bound to the promoters of nearly half of the tested

261 TFs (66 TFs) and then indirectly regulated numerous downstream genes.

262 We examined the 125 TFs that were analysed through ChIP-seq and exhibited high

263 co-association scores. We determined the co-association patterns among these

264 regulators (**Figure 2a**) and classified them into four clusters, denoted as C1 to C4.

265 Notably, C1 and C4 contained higher proportions of bottom-level TFs. This finding

266 indicated that the bottom-level TFs, which were more easily regulated, tended to

267 cooperate with downstream genes and other intra-level TFs. When comparing the

268 co-association scores of TF pairs, we observed a stronger tendency toward

269 cooperation among lower-level TFs, especially bottom-level TFs. In particular, 35

270 bottom-level TFs in C4 (83%) exhibited coregulation at the same peak locations with

271 high co-association scores. For instance, PhnF and PSPPH4692 co-bound to three

272 target genes (PSPPH4117, PSPPH4216 and PSPPHB0021), with co-association

273 scores as high as 0.92. This co-binding relationship between all TF pairs was defined

274 as an indirect interaction. In contrast to the trend observed for direct interactions and
275 cooperativity, we observed stronger correlations between top-level TFs and TFs
276 situated at higher levels (top and middle levels; **Figure 1b**). For example, the
277 top-level TFs PSPPH3798 and CysB co-bound to the promoter region of *flhB*, which
278 encodes a flagellar biosynthesis protein. We found a similar co-association pattern
279 among bottom-level TFs. However, middle-level TFs displayed the weakest
280 correlations internally, indicating that regulation by higher-level TFs was distributed
281 across different regions in targeted promoters. For instance, we observed that
282 PSPPH3798 bound upstream of *flhB*, whereas the peak of CysB was located in the
283 overlapStart region.

284 We found that the correlation between bottom-level TFs was weaker in C3 than in the
285 other clusters. To further explore the DNA-binding characteristics of all TFs within
286 the same cluster, we investigated the peak features of the LysR-family TF MexT in C3
287 as an example. The analysis of the peak locations of MexT demonstrated that MexT
288 showed closer co-association relationships with top-level TFs (**Figure 2b**). Within the
289 binding peaks of MexT, the target genes PSPPH3643 (LysR family TF), PSPPH2618
290 (sulphite reductase (NADPH) haemoprotein, CysI) and PSPPH2411 (hypothetical
291 protein) displayed high co-association scores with other TFs. UpSet plots
292 demonstrated that 12 TFs in C3 bound to a highly overlapping region of CysI (**Figure**
293 **2c**). These results suggest that these genes were prioritised for co-regulation by MexT.
294 To further explore the binding features in C3, we determined the motifs of 11 TFs
295 based on their binding sequences obtained via ChIP-seq using MEME(Bailey et al.,

296 2009), even though the binding motif of MexT was previously investigated(Tian et al.,
297 2009). We identified a 15-bp consensus motif (ATN11AT) throughout the 11 analysed
298 TFs (**Figure 2d**), demonstrating a high degree of consensus in co-association patterns
299 among TFs in *P. syringae*.

300 **Virulence-associated pathways were primarily regulated by top-level TFs in *P.***
301 ***syringae***

302 Because the pathogenicity of *P. syringae* mainly depends on T3SS and other
303 virulence-associated pathways(He, 1998), we particularly focused on TFs that bind to
304 numerous virulence-related genes. Seven pathways manipulate the virulence of *P.*
305 *syringae*(Huang et al., 2022; Xie, Shao, & Deng, 2019), namely T3SS, biofilm
306 production, motility, nucleotide-based secondary messenger function, quorum sensing
307 (QS), phytotoxin production and siderophore production. To comprehensively
308 investigate the transcription regulatory mechanism underlying the virulence of *P.*
309 *syringae*, we calculated the hierarchical heights of TFs involved in virulence
310 regulation and the virulence genes modulated by them. This analysis provided insights
311 into the organisation of the virulence regulatory network in *P. syringae*, where
312 virulence-involved TFs were categorised into three tiers (**Figure 3a**).

313 We found three transcriptional regulatory channels governing virulence regulation in
314 *P. syringae*. The first channel was the direct trigger, which has been extensively
315 studied in previous studies and is referred to as the ‘one-step trigger’ here(Fan et al.,
316 2020). These TFs are recognised as master regulators that directly respond to

317 biological events without additional intermediaries(Chan & Kyba, 2013). In our
318 previous study, we identified TrpI, RhpR, GacA and PSPPH3618 as master regulators
319 in T3SS and 16 master regulators in other virulence pathways(Fan et al., 2020). In
320 line with this definition, we recognised 35 TFs (PSPPH4644, AlgQ, PSPPH1100,
321 CysB, PSPPH2555, PSPPH0239, PSPPH1762, Irp, PSPPH1951, PSPPH2193,
322 PSPH3504, PSPPH1435, PSPPH2983, PSPPH0700, PSPPH5132, PSPPH1776,
323 PSPPH4700, SfsA, PSPPH3522, PSPPH3268, PSPPH4356, PSPPH3798,
324 PSPPH4638, PSPPH4127, Lon, PSPPH4324, PSPPH4673, MexT, PSPPH4844,
325 PSPPH2214, PSPPH0755, PSPPH4012, PSPPH4920, Anr and PSPPH2476) that
326 participate in various virulence pathways. More than 68% of these TFs (24 of 35 TFs)
327 were at the top levels, indicating that the virulence of *P. syringae* is primarily
328 regulated by top-level TFs. Among these TFs in the network, the top-level TFs
329 PSPPH1951, PSPPH2193 and PSPPH3268 were found to have abundant
330 virulence-associated target genes based on ChIP-seq results. The de novo motif
331 analysis of their peak sequences revealed the presence of three head-to-head motifs: a
332 17-bp motif (AT-N13-AT) for PSPPH1951, a 15-bp motif (ATC-N9-GAT) for
333 PSPPH2193 and a 10-bp motif (AC-N6-GT) for PSPPH3268 (**Figure 3b–d, Figure**
334 **S4a–c**).

335 **Three master TFs were identified to participate in virulence**

336 To further verify the biological functions of these three uncharacterised TFs, we first
337 purified the TF proteins and performed an electrophoretic mobility shift assay (EMSA)
338 to confirm their direct interactions with key virulence genes *in vitro*. Next, we

339 generated TF deletion strains to detect the transcription levels of target genes. We
340 found that PSPPH1951 directly regulated multiple T3SS genes, including
341 *hrpRhopAE1* and *hopAH2* (**Figure 3b**). Among them, the expression of *hrpR* in
342 Δ PSPPH1951 was significantly increased by more than four-fold compared with the
343 WT, suggesting that PSPPH1951 acts as a repressor of *P. syringae* T3SS (**Figure 3b**).
344 In addition, PSPPH1951 was found to bind to the promoters of type IV pili genes
345 (*pilG*, *pilF* and *pilZ*; **Figure S4d**).

346 *P. syringae* enhances its ability to infect the host by increasing bacterial motility (Buell
347 et al., 2003). ChIP-seq data (*in vivo*) and EMSA (*in vitro*) results showed that
348 PSPPH2193 interacted with the promoters of motility-related genes, such as *fleQ*
349 (encoding a flagellar regulator) and *flhF* (encoding a flagellar biosynthesis regulator;
350 **Figure 3c**). RT-qPCR results indicated that *fleQ* and *flhF* were downregulated
351 two-fold in Δ PSPPH2193 compared with the WT. As expected, Δ PSPPH2193
352 exhibited weaker motility than the WT and complementary strain in King's B medium
353 (**Figure 3c**), indicating that PSPPH2193 serves as an activator of motility in *P.*
354 *syringae*.

355 PSPPH3268 was found to influence the pathogenicity of *P. syringae* by regulating
356 multiple virulence-related pathways. During the initial stage of *P. syringae* infection,
357 the bacteria produce biofilm components, including extracellular polysaccharides
358 (EPSs), type IV pili and other highly viscous compounds. These components help the
359 bacteria to establish colonies, providing protection against the host's immune
360 defences and antimicrobial agents (Whitchurch, Tolker-Nielsen, Ragas, & Mattick,

361 2002). We found that PSPPH3268 strongly interacted with the promoters of key genes
362 involved in biofilm production such as *hrpR*, the alginate biosynthesis gene *alg44* and
363 the type IV pilus assembly gene *pilM* (**Figure 3d**). Furthermore, the transcription
364 levels of *alg44*, *algX* (encoding the alginate biosynthesis protein) and *pilM* were
365 markedly enhanced in Δ PSPPH3268. This resulted in enhanced biofilm formation and
366 EPS production when the PSPPH3268 gene was deleted and then restored when
367 PSPPH3268 was expressed (**Figure 3d**). These results demonstrated that PSPPH3268
368 acts as a master regulator in various virulence-related pathways. In addition, we
369 identified that the TF PSPPH3798 binds to the promoters of flagellar-related genes
370 (*fliK*, *fliE*, *fliD* and *fleQ*; **Figure S4e**).

371 In addition to the ‘one-step trigger’ mechanism, we found that TFs also regulate
372 downstream genes through one or two other TFs at different levels, which were
373 regarded as ‘one jump-point trigger’ and ‘two jump-point trigger’. For example,
374 PSPPH2555 indirectly influenced biofilm formation (*algD*), motility (*fleQ*), T3SS
375 (*hrpR*), QS (*ahlR* and *secE*) and phytotoxin production (*aprD*) by directly regulating
376 the bottom-level TF PSPPH4920 (**Supplementary Table 3**). PSPPH3504 was found
377 to be involved in a
378 PSPPH3504-Lon/PSPPH4324/PSPPH4844-PSPPH0755/PSPPH4920/PSPPH4012-tar
379 get gene pathway (**Figure S4f, Supplementary Table 3, ‘/’ represents sibling nodes
380 and ‘-’ represents downward regulation**). Among these TFs, PSPPH0755,
381 PSPPH4920 and PSPPH4012 are considered key performer TFs because they mediate
382 most of the transcription regulatory signals from multiple TFs. We also found reverse

383 regulatory pathways in our network. For example, the middle-level TF PSPPH4673
384 was found to directly regulate the top-level TF PSPPH4700 and then indirectly
385 control the transcription of many virulence-related genes through regulating the
386 bottom-level TFs PSPPH0755, PSPPH4920, Anr, and PSPPH2476 (**Supplementary**
387 **Table 3**). In summary, TFs regulate the pathogenicity of *P. syringae* through diverse
388 pathways, either by directly binding to target genes or indirectly controlling other
389 TFs.

390 **Systematic mapping of TF targets revealed key metabolic regulators in *P.*** 391 ***syringae***

392 In addition to enhancing pathogenicity and resisting host defences, *P. syringae* adjusts
393 its metabolic activities to survive in unpredictable environments (Rico, McCraw, &
394 Preston, 2011). To comprehensively understand metabolic regulation in *P. syringae*,
395 we constructed a hierarchical network that includes key regulators and the genes they
396 trigger, similar to the virulence hierarchical network. We focused on eight metabolic
397 pathways, namely amino acid biosynthesis, DNA replication, ATP-binding cassette
398 (ABC) transportation, oxidative phosphorylation, tricarboxylic acid (TCA) cycle,
399 RNA polymerase, phosphonate metabolism and methyl-accepting chemotaxis (**Figure**
400 **4a**). Compared with the virulence network shown in Figure 3A, we identified more
401 TFs involved in metabolic regulation, many of which exhibited numerous interactions
402 with genes related to oxidative phosphorylation (178 binding peaks) and the TCA
403 cycle (154 binding peaks; **Figure S5a–c**). In a previous study, 12 master regulators
404 were reported to control various metabolic pathways, including LexA1, PSPPH3004

405 and PSPPH1960, involved in reactive oxygen species (ROS) resistance(Fan et al.,
406 2020). RhpR participates in several metabolic pathways, such as ABC transporters
407 and oxidoreductase activity(Shao et al., 2019). Lon is involved in glucokinase and
408 oxidoreductase activity(Hua et al., 2020). MgrA, GacA, PilR, PsrA, RpoN, CvsR,
409 OmpR and CbrB2 participate in oxidation resistance, amino acid transportation and
410 other metabolic pathways(Shao et al., 2021). Here, we identified 111 TFs regulating
411 these eight metabolic pathways. Similar to the aforementioned virulence network,
412 three transcriptional regulatory channels were observed for the metabolic pathways.
413 To provide a detailed view of metabolic regulation in *P. syringae*, we counted the
414 number of functionally annotated genes related to each pathway and calculated the
415 proportion of targets for each TF, highlighting key regulators in these eight metabolic
416 pathways using radar plots (**Figures 4b and S6a**).

417 We found that the TFs CysB and PSPPH3268 regulate all eight metabolic pathways,
418 whereas the TFs PSPPH1951, PSPPH3798, PSPPH3504 and PSPPH4700 were
419 predicted to regulate seven metabolic pathways. Notably, the TF PSPPH0755 was
420 found to bind to the promoters of PSPPH5210 (encoding ATP synthase F0F1 subunit
421 delta) and PSPPH3109 (encoding the NADH dehydrogenase subunit A NuoA). The
422 monomer motif (CTGAA) of PSPPH0755 was identified through MEME analysis.
423 The interactions in these two metabolic pathways were confirmed through EMSA
424 (**Figure S6b**). The TF PSPPH3798 was predicted to bind to the promoters of genes in
425 two pathways, including PSPPH3881 (encoding the methyl-accepting chemotaxis
426 protein WspA) and PSPPH5119 (encoding the phosphate transport system regulatory

427 protein PhoU). The 15-bp binding motif of PSPPH3798 was determined to have a
428 head-to head orientation (ATCG-N7-CGAT). EMSA results confirmed these
429 interactions (**Figure S6c**). In addition to the TF PSPPH3798, the TF PSPPH4638 had
430 a binding site in the promoter region of the PSPPH3881 gene. PSPPH4638 was also
431 predicted to interact with PSPPH0550 that encodes phosphoserine phosphatase SerB.
432 The 8-bp monomer motif (ATTTTCAA) of PSPPH4638 was identified, and the
433 binding interactions were confirmed through EMSA (**Figure S6d**).

434 In yeast, TFs in a functional category appear to bind to genes in the same
435 category (Simon et al., 2001), and we observed a similar pattern in *P. syringae*. For
436 example, TF Irp, a key regulator in the methyl-accepting pathway, bound to the
437 promoters of PSPPH3798, PSPPH4638 and PSPPH4700, which were also identified
438 as key regulators in the same pathway (**Figure 4c**). In addition, we found that TFs
439 from different categories often bound to the promoters of TFs responsible for other
440 cellular processes. For example, key regulators controlling oxidative phosphorylation
441 (highlighted in red and blue; PSPPH1951, PSPPH2193, PSPPH2832, PSPPH3268,
442 PSPPH3504, PSPPH3798, PSPPH4638, PSPPH4673 and PSPPH4700) bound to TFs
443 playing key roles in the methyl-accepting pathway (highlighted in brown and blue;
444 PSPPH1951, PSPPH3268, PSPPH3504, PSPPH3798, PSPPH4638, PSPPH4700 and
445 Irp; **Figure 4d**). These results demonstrate that many regulatory processes are often
446 achieved through coregulation by a series of multifunctional TFs throughout the
447 network, enabling *P. syringae* to coordinate transcriptional regulation processes across
448 multiple cellular processes.

449 **TFs indicated large functional variability across different pathovars in *P.***

450 *syringae*

451 Although TF functions exhibit both inter- and intra-species variability, most previous
452 studies on TFs have focused on the molecular mechanism of a single strain (Galardini
453 et al., 2015). To investigate the regulatory mechanism of TFs across different strains
454 of *P. syringae*, we selected four model strains: *P. syringae* pv. *syringae* 1448A, *P.*
455 *syringae* pv. *tomato* DC3000, *P. syringae* pv. *syringae* B728a and *P. syringae* pv.
456 *actinidiae* C48. We used the genome of 1448A as a reference and conducted a
457 homology analysis of 1448A protein sequences with those of the other three strains.
458 We determined a high proportion of homologous proteins in the three strains (4983 in
459 DC3000, 4982 in B728a and 4984 in C48; **Supplementary Table 4**). Across the four
460 strains, all 301 annotated TFs were present. We selected five TFs (Irp, PSPPH2193,
461 PSPPH3122, PSPPH4127 and OmpR) to construct TF-overexpressing strains in
462 DC3000, B728a and C48 before performing CHIP-seq analysis. We identified the
463 binding sites of all these TFs in the four strains and found divergent binding
464 preferences for the same TFs in different strains. Most target genes of each TF in one
465 or two strains were unique. In particular, Irp bound to 19 target genes that were
466 conserved in all four tested strains, including *purB* (encoding adenylosuccinate lyase),
467 *cceA2* (encoding the chemotaxis sensor histidine kinase) and *gidA* (encoding the
468 tRNA uridine 5-carboxymethylaminomethyl modification protein). Four highly
469 conserved target genes were also found to directly interact with PSPPH4127.
470 Evolutionary analysis of the binding peaks of TFs suggested high binding specificity

471 and varying levels of conservation of these TFs in the tested strains (**Figure 5a**).

472 In addition to the intersection between 1448A and C48, we observed differences
473 between the target genes of all five TFs (**Figure 5b**) and the peak locations (TF target
474 interactions; **Figure 5c**) in these four pathovars. The inconsistency between the
475 number of targets and peaks suggested that some target genes were regulated by at
476 least one different TF in these four strains, which is similar to regulation in *P.*
477 *aeruginosa* (Trouillon et al., 2021). To confirm the presence of target genes regulated
478 by the same TF or different TFs in various strains, we compared the peak locations of
479 *gidA* and *rpoD* (RNA polymerase sigma factor) as two examples. TF Irp was found to
480 bind to the promoter of *gidA* in four strains (**Figure 5d**) and had 19 conserved target
481 genes in these strains (**Supplementary Table 4**). In addition, PSPPH4127 had four
482 conserved target genes (*rpoD*, PSPPHPPH_1001, PSPPHPPH_1998 and
483 PSPPHPPH_5016) in all four strains (**Figure 5e**). These results showed that Irp and
484 PSPPH4127 exhibited higher functional conservation than the other three TFs. We
485 also found that PSPPH2193 in 1448A and Irp in DC3000 bound to the promoter of
486 *rpoD* (**Figure 5e**). Differences in the regulation of the same targets by different TFs
487 were also observed in more than 1,500 target genes, suggesting the potential diversity
488 of the transcriptional regulation of TFs in our network.

489 **Topological modularity of the transcription regulatory network exhibited**
490 **various functions in biological processes in *P. syringae***

491 Complex networks in nature often exhibit topological and/or functional

492 modularity(Dittrich, Klau, Rosenwald, Dandekar, & Müller, 2008; Olesen, Bascompte,
493 Dupont, & Jordano, 2007). We used a partitioning algorithm (with a resolution of 0.9)
494 to classify network elements into different subsets using Gephi. Our analysed TFs and
495 their target genes were divided into 16 modules, each represented by a different
496 colour (**Figure 6a**). Each module contained 2.7% to 12.1% of the total elements and
497 exhibited correlations with each other. We found that almost all nodes in the network
498 had connections both within and between modules, indicating that the 16 modules
499 were not isolated and contributed to extensive information flow throughout the
500 network to regulate transcription in *P. syringae* (**Figure 6b**). Module 12 appeared to
501 play a central role in facilitating large information flows with other modules. Module
502 15 also exhibited transcriptional information exchanges both between modules and
503 within the same module.

504 Among the 16 modules, Module 2 was involved in most nodes (443 elements,
505 including 41 TFs and 402 target genes). Module 3 contained the least TFs (181
506 elements, including two TFs and 179 target genes). To investigate the potential
507 correlation between topological modularity and biological functions, we performed
508 GO term and KEGG pathway enrichment analysis for each module. As expected, 15
509 modules were enriched in specific biological functions (**Figure 6c and d**). In some
510 modules, hundreds of elements were assessed. For example, genes in Module 2 (443
511 nodes), Module 12 (327 nodes) and Module 14 (432 nodes) were mainly enriched in
512 the regulation of transcription and DNA binding (**Figure 6c**). DksA, a TF in Module 2,
513 played a key role in regulating transcription-coupled DNA repair(Meddows, Savory,

514 Grove, Moore, & Lloyd, 2005) and also participated in oxidative phosphorylation,
515 amino sugar and nucleotide sugar metabolism and RNA degradation in *P. syringae*.
516 Twenty TFs (such as CapA, CapB, CysB, FruR and MarR) classified in Module 12
517 were identified to be involved in transcriptional regulation. The TF PSPPH3798
518 located in Module 14 was observed to be involved in flagellar assembly, and these
519 interactions were confirmed by EMSA (**Figure S4b**). The genes in Module 4 were
520 enriched in oxidoreductase activity, and our analysis revealed that the TF PSPPH0755
521 played a role in regulating the oxidative phosphorylation pathway (**Figure 6d and**
522 **S6b**). MexT in Module 5, which was previously associated with motility in *P.*
523 *syringae* (Kawakita et al., 2012), was found to participate in in biofilm formation and
524 QS pathways in our study. In addition, we not only identified the crucial roles of the
525 TFs PSPPH1951 (Module 6), PSPPH2193 (Module 11) and PSPPH3268 (Module 15)
526 in T3SS pathways, bacterial motility and biofilm formation, respectively (**Figure 3b–**
527 **d**), but also reported their potential biological functions in aminoacyl-tRNA
528 biosynthesis (PSPPH1951), RNA degradation (PSPPH2193) and catalytic activity
529 (PSPPH3268) (**Figure 6d**). Our results allowed us to identify the potential regulators
530 of specific pathways and perform functional predictions for hypothetical proteins in *P.*
531 *syringae*. For instance, PSPPH1503 in Module 15, which encodes a hypothetical
532 protein, was possibly correlated with glycerophospholipid metabolism.

533 Discussion

534 Most microbial studies on genome-wide transcriptional regulatory network focus on *S.*
535 *cerevisiae* and *E. coli*, which reveal the principles of architecture and interactions of
536 their regulatory networks. The analysis of the transcription regulatory associations in
537 *S. cerevisiae* mainly rely on the databases such as YEASTRACT (YEAsT Search for
538 Transcriptional Regulators And Consensus Tracking)(Teixeira et al., 2018). In *E.coli*,
539 relative complete transcriptional regulatory network has been generated through
540 integrating three different data sources (RegulonDB, Ecocyc and TRN-SO)(Ma et al.,
541 2004). However, few study has yet comprehensively evaluated TFs in other
542 prokaryotic species throughout a genome(Ishihama et al., 2016). In this study, we
543 successfully generated the most complete transcriptional regulatory network and data
544 source, which profiled the transcriptional regulatory features of both the
545 aforementioned 100 TFs and an additional 170 TFs in *P. syringae* through ChIP-seq.
546 By mapping the TF-target hierarchical regulatory networks, we identified several
547 novel master regulators involved in significant biological processes. Furthermore, our
548 evolutionary analysis and assessment of the topological functional modularity of TFs
549 and their respective targets revealed the evolutionary conservation and functional
550 diversity of TFs in *P. syringae*.

551 Although transcriptional regulatory networks are considered conserved(Perez &
552 Groisman, 2009), many studies reveal highly functional variability of TFs in inter-
553 and intra-species(Galardini et al., 2015). These observed diversities between different
554 strains of the same species mainly result from the expression levels of TFs, contents

555 of target genes, and differences of binding sequences(Trouillon et al., 2021). In our
556 study, we observed large differences of DNA-binding characteristic of TF Irp between
557 the C48 strain and the DC3000 strain. The functional diversity of TFs may arise from
558 the large difference in the contents of target genes and TFs, which are regarded as the
559 main determinant of transcriptional regulatory(van Duin, Krautz, Rennie, &
560 Andersson, 2023), although Irp display high homology in these pathovars.

561 Collaborations between TFs at higher levels (top and middle) were enriched, a pattern
562 similar to the tendencies observed in human TFs(Gerstein et al., 2012). In particular,
563 TT TF pairs exhibited a greater degree of cooperative gene regulation, whereas TB TF
564 pairs accounted for nearly half of direct interactions within all communications.
565 Furthermore, we observed that both direct physical regulation and cooperative
566 interactions were the least common among MM TF pairs. By contrast, in humans,
567 direct regulation tends to occur between TT or TM TF pairs. Furthermore, interactions
568 between TFs, in any form, within human and yeast transcriptional regulatory
569 networks are more likely to appear between middle TFs, which act as information
570 transfer centres(Bhardwaj, Yan, & Gerstein, 2010). This may be attributed to the
571 higher abundance of bottom-level TFs than higher-level TFs observed in prokaryotic
572 microorganisms, a pattern also found in *E. coli*(Bhardwaj et al., 2010). This finding
573 indicates that the bottom-level TFs that are more likely to be regulated are
574 evolutionarily preferred in multicellular eukaryotic organisms. However, in *E. coli*,
575 the deficient bottom-level TFs are more commonly co-associated with other
576 same-level TFs. When comparing co-associated TF pairs and the cooperativity of TFs,

577 we observed a distinct and inverse relationship in *P. syringae* compared with yeast or
578 *E. coli* (**Figure 1B**). The enriched cooperativity of bottom-level TFs with high
579 co-associated scores indicated that these bottom-level TFs preferred to coregulate
580 target genes by binding to the same peak locations. Notably, seven TFs without
581 correlations with other TFs appeared to independently participate in biological
582 processes. These findings not only shed light on the inherent properties of direct
583 regulation and co-association across various species but also indicate the unique
584 characteristics of *P. syringae* to response to dynamic environmental variations.

585 The fundamental units of a transcriptional regulatory network are positive and
586 negative loops. For a more comprehensive description, these regulatory units can be
587 classified into six submodules, namely autoregulation, multicomponent loops,
588 feedforward loops, single-input, multi-input and regulator chain(Lee et al., 2002). In
589 yeast, only 10 TFs were found to autoregulate themselves, whereas the majority of
590 regulatory units among 116 TFs in *E. coli* exhibit autoregulation(Thieffry, Huerta, Pé
591 rez-Rueda, & Collado-Vides, 1998). Similarly, we identified 92 autoregulators in our
592 transcriptional regulatory network, which may function as auto-inhibitors in *E. coli*.
593 Feedforward loops are highly prevalent in prokaryotic transcriptional regulatory
594 networks, such as human and yeast(Gerstein et al., 2012; Lee et al., 2002). The 696
595 feedforward loops (M13) identified in our study highlighted this cooperative
596 regulation of TFs in response to small signals in prokaryotic species. This enriched
597 submodule is regarded as a temporal switch that provides constant feedback to
598 respond rapidly to signal impacts(Shen-Orr et al., 2002). Multistep regulation assists

599 master regulators in enhancing the initial information flow(Goldbeter & Koshland Jr,
600 1984). Unlike M3, the most abundant module (868) in the human TF regulatory
601 network, we observed that M1 was the most prevalent module (24,479) in the *P.*
602 *syringae* transcriptional regulatory network. This finding indicates that *P. syringae*
603 prefers achieving transcriptional regulation through few TFs to ensure rapid
604 transmission and response to environmental signals.

605 Despite more than 20 years of research, our understanding of the global regulatory
606 network in the plant pathogen *P. syringae* remains inadequate. Our previous studies
607 reported 16 key virulence-related regulators(Shao et al., 2021), 25 master
608 virulence-related regulators(Fan et al., 2020) and seven global regulators acting as
609 TCSs(Xie et al., 2022). However, the regulatory relationships and functional crosstalk
610 among all TFs in *P. syringae* remain unknown. In this study, we integrated all
611 available interaction information concerning almost all TFs in *P. syringae* and mapped
612 the first comprehensive transcriptional regulatory network in this plant pathogen
613 (**Figure 7**). This network offers a global view of the multiple functions of TFs in *P.*
614 *syringae*. We also identified 35 vital TFs that participate in virulence pathways and
615 111 key TFs involved in metabolic pathways across the global transcriptional
616 regulatory network in *P. syringae*. This analysis uncovered new functions of
617 previously characterised TFs, such as MexT. In addition to the previously reported
618 *mexEF-oprN* operon,(Sawada et al., 2018) this study identified *fleQ* (a flagellar
619 regulator) and *shcF* (a type III chaperone protein) as the targets of MexT. In addition,
620 we investigated the functional evolution and potential intra-species variability of TFs

621 in *P. syringae*, demonstrating the functional diversity of TFs among *P. syringae*
622 species during their long course of evolution. Based on the aforementioned results, we
623 established the *P. syringae* transcriptional regulatory network (PSTRnet) database,
624 which contains detailed binding peak information for all TFs in *P. syringae*
625 (https://jiadhuang0417.shinyapps.io/PSTF_NET/). This database serves as a valuable
626 platform for presenting, searching and downloading regulatory information of
627 transcription in *P. syringae*.

628 We observed crosstalk between not only TFs but also various pathways. For example,
629 the TF PSPPH4700 directly regulated *fleQ* and *hrpR* and indirectly regulated *fleQ* and
630 *hrpR* through the PSPPH4700/PSPPH4324/PSPPH0755 cascade. Feedforward loop
631 modules are usually coherent, meaning that the direct effect of downstream TFs has
632 the same regulatory direction (positive or negative) as the indirect effect of upstream
633 TFs (Shen-Orr et al., 2002). This observation enhanced our understanding of the
634 influence of TFs in the network on their target genes based on the identified effects of
635 other TFs. Furthermore, the TF PSPPH4700 was identified to bind to the promoters of
636 genes involved in seven metabolic pathways, namely the TCA cycle, oxidative
637 phosphorylation, methyl-accepting, amino acid biosynthesis, RNA polymerase, ABC
638 transportation and DNA replication. Our results proved the possibility of crosstalk
639 between different pathways in *P. syringae*.

640 Taken together, our study provides comprehensive insights into the DNA-binding
641 characteristics and potential regulatory pathways of almost all annotated TFs in *P.*
642 *syringae*. The global transcriptional regulatory network can not only contribute to the

- 643 development of novel drugs to combat *P. syringae* infections but also advance
- 644 research on the molecular mechanisms of TFs in other pathogens.

645 **Materials and Methods**

646 **Bacterial strains, culture media, plasmids, and primers**

647 The bacterial strains, plasmids and primers used in this study are listed in
648 **Supplementary Table 5**. *P. syringae* 1448A, DC3000, B728a and C48 strains were
649 grown at 28 °C in King' B (KB) medium shaking at 220 rpm or KB agar plates(King,
650 Ward, & Raney, 1954). *E. coli* BL21(DE3) or DH5 α strains were grown at 37 °C in
651 Luria-Bertani medium (LB) shaking at 220 rpm or on LB agar plates. Antibiotics used
652 for *P. syringae* 1448A, DC3000 and B728a wide type (WT) strains and mutants were
653 rifampicin at 50 μ g/ml; *P. syringae* 1448A, DC3000 and B728a overexpression strains
654 were rifampicin at 50 μ g/ml and spectinomycin at 50 μ g/ml, C48 overexpression
655 strains were spectinomycin at 50 μ g/ml; *P. syringae* 1448A strains with
656 pK18mobsacB plasmids for mutant construction were rifampicin at 50 μ g/ml and
657 kanamycin at 50 μ g/ml. Antibiotics used for *E. coli* with pET28a plasmids for protein
658 purification, and pK18mobsacB plasmids were kanamycin at 50 μ g/ml; *E. coli* with
659 pHM1 plasmids for overexpression strain construction were spectinomycin at
660 50 μ g/ml.

661 **Construction of overexpression strains and mutants**

662 Overexpression strains and mutants of *P. syringae* were constructed as previously
663 described(Kvitko & Collmer, 2011; Shao et al., 2021). In brief, for overexpression
664 strain, the open reading frame (Caillet, Baron, Boni, Caillet-Saguy, & Hajnsdorf) of
665 each TF-coding gene was amplified by PCR from *P. syringae* genome and cloned into

666 pHM1 plasmid. The ligated fragments were inserted into the digested pHM1 plasmids
667 (*Hind*III) using ClonExpress MultiS One Step Cloning Kit (Vazyme). The
668 recombinant plasmids were transformed into the *P. syringae* wild-type strain. The
669 single colonies were confirmed by western blot. For mutants, the upstream (~1500-bp)
670 and downstream (~1000-bp) fragments of TF ORF were amplified by PCR from the *P.*
671 *syringae* genome and digested with *Xba*I respectively, and then ligated by T4 DNA
672 ligase (Jumper et al.). The ligated fragments were inserted into the digested
673 pK18*mobsacB* plasmids (*Xba*I) using ClonExpress MultiS One Step Cloning Kit
674 (Vazyme). The recombinant plasmids were transformed into the *P. syringae* wild-type
675 strain. The single colonies were selected to the sucrose plates, and further screened in
676 two kinds of KB plates (with kanamycin/rifampin and with rifampin) concurrently.
677 The single colonies losing kanamycin resistance were further verified by real-time
678 quantitative PCR (RT-qPCR) to detect the mRNA level of corresponding TF genes.

679 **ChIP-seq analyses**

680 As previous description(Blasco et al., 2012), the overexpression strains of
681 corresponding TFs and *P. syringae* WT with empty pHM1 plasmid was cultured in 30
682 mL KB medium to OD₆₀₀=0.6. Bacterial cultures were cross-linked with 1%
683 formaldehyde for 10 min at 28°C and then the reaction was stopped by the addition of
684 125 mM glycine for 5 min. The centrifugated bacteria were washed twice with Tris
685 Buffer (20 mM Tris-HCl [pH 7.5] and 150 mM NaCl) and washed again with IP
686 Buffer (50 mM HEPES–KOH [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton
687 X-100, 0.1% sodium deoxycholate, 0.1% SDS, and mini-protease inhibitor cocktail).

688 The centrifuged bacteria were preserved at 80°C or continued for the next
689 experiments. The bacteria were subjected with IP Buffer and then sonicated to pull
690 down the DNA fragments (150-300-bp). The supernatant was the DNA-TF-HA tag
691 complex and used as IP samples. IP experiments and control sample were incubated
692 with agarose conjugated anti-HA antibodies (Sigma) in IP Buffer. The complex of
693 DNA-TF-anti-HA agarose was applied to washing, crosslink reversal, and
694 purification(Blasco et al., 2012). The 150-250-bp DNA fragments were selected for
695 library construction. The libraries were sequenced using the HiSeq 2000 system
696 (Illumina). Two biological replications have been performed for all ChIP-seq
697 experiments. ChIP-seq reads were mapped to the *P. syringae* 1448A genome
698 (NC_005773.3), DC3000 (NC_004578.1), B728a (NC_007005.1) and C48
699 (NZ_CP032631.1) using Bowtie2 (version 2.3.4.3)(Zhang et al., 2008). Subsequently,
700 binding peaks ($q < 0.01$) were identified using MACS2 software (version 2.1.0). The
701 enriched loci for each TF were annotated using the R package ChIPpeakAnno
702 (version 3.18.2)(L. J. Zhu et al., 2010).

703 **Electrophoretic mobility-shift assay**

704 DNA probes were amplified from *P. syringae* 1448A genome by PCR using primers
705 listed in Supplementary Table 5. The probes (20 ng) were mixed with various
706 concentrations of proteins in 20 μ L of gel shift buffer (10 mM Tris-HCl, pH 7.4, 50
707 mM KCl, 5 mM MgCl₂, 10% glycerol). After incubation at room temperature for 20
708 min, the samples were analyzed by 6% native polyacrylamide gel electrophoresis (90
709 V for 90 min for sample separation). The gels were subjected to Gel Red dye (Tiangen

710 Biotech) for 5 min and photographed by using a gel imaging system (Bio-Rad). The
711 assay was repeated at least three times with similar results.

712 **RT-qPCR**

713 The RT-qPCR primers used are shown Supplementary Table 5 in the Supplemental
714 Information. The cultured bacteria were grown to $OD_{600}=0.6$ and the total RNA were
715 purified with Bacteria Total RNA Isolation Kit (Sangon Biotech). The RNA
716 concentrations were measured using a NanoDrop 2000 spectrophotometer
717 (ThermoFisher). cDNA synthesis was performed using HiScript III RT SuperMix
718 (Vazyme, China). RT-qPCR was performed with a SuperReal Premix Plus (SYBR
719 Green) kit (Vazyme, China) according to the manufacturer's instructions. The
720 reactions used 100 nM primers and were run for 40 cycles at 95°C for 30 s and 95°C
721 for 10 s, and at 60°C for 30 s. The fold change represents relative expression level of
722 mRNA, which can be estimated by the values of $2^{-(\Delta\Delta Ct)}$. The relative expression of
723 target genes in WT was set to 1. All the reactions were conducted with three repeats.

724 **Motility assay**

725 The motility assay was performed based on our previous study(Shao et al., 2021).
726 Swimming plates were KB agar plates containing 0.3% agar (MP Biomedicals, UK)
727 and rifampicin at 25 $\mu\text{g}/\text{ml}$. Overnight bacterial cultures were inoculated on
728 swimming plates as 2 μL aliquots and incubated at room temperature for 3-5 days.
729 Finally, the diameter of motility trace represented the swimming motility of *P.*
730 *syringae* strains. Photographs were taken by using the Bio-Rad imaging system. The

731 assay was repeated at least three times with similar results.

732 **Congo red assay**

733 Congo red assay was performed as previous study(Shao et al., 2021). Congo red
734 plates were KB agar plates containing 1.0% agar (MP Biomedicals, UK) and
735 rifampicin at 25 µg/ml. Overnight bacterial cultures were inoculated on Congo red
736 plates as 2 µL aliquots and incubated at 28°C. The colony staining was photographed
737 after 5-7 days. The assay was repeated at least three times with similar results.

738 **Biofilm formation assay**

739 Biofilm formation assay was performed as previously describe(Shao et al., 2019).
740 Overnight bacterial cultures were transferred to a sterile 10 mL borosilicate tube
741 containing 2 ml KB medium with rifampicin (25 µg/ml) with the original
742 concentration $OD_{600} = 0.1$. The cultures grew at room temperature for 36 hr without
743 shaking. 0.1% crystal violet was used to stain the biofilm adhered to the tube for 20
744 min without shaking. Tubes were washed for more than three times with distilled
745 deionized water gently and other components on the tube loosely was washed off. The
746 tubes were dry and photographed. The remaining crystal violet was fully dissolved in
747 1 ml 95% ethanol with constantly shaking and measured its optical density at 590 nm
748 (Biotek microplate reader). The assay was repeated at least three times with similar
749 results.

750 **Network and functional enrichment analysis**

751 Network analyses were performed on Gephi 0.10. Functional annotations were

752 retrieved from the *Pseudomonas* database and GO functional enrichment analyses and

753 KEGG analysis were performed using DAVID v6.8.

754 **Statistical analysis**

755 Two-tailed Student's *t* tests were performed using Microsoft Office Excel 2010.

756 * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ and results represent means \pm SD. All

757 experiments were repeated at least twice.

758 **Data Availability**

759 Sequencing data have been deposited and publicly available in Gene Expression
760 Omnibus (GEO) under accession number GSE247395. Source data contain the
761 numerical data used to generate the figures of EMSA. Hierarchical information and
762 functional categories of TFs are available in **Supplementary Table 1-3**. Evolutionary
763 details are provided in **Supplementary Table 4**. Primers and strains used in this paper
764 are provided in **Supplementary Table 5**.

765 **Funding**

766 This study was supported by grants from the National Natural Science Foundation of
767 China (31670127 and 31870116 to X.D.), General Research Funds of Hong Kong
768 (21103018, 11101619, and 11102720 to X.D.). The funders had no role in study
769 design, data collection, interpretation, or the decision to submit the work for
770 publication.

771 **Acknowledgments**

772 X.D., Y.S., J.W. and J. H. conceived the project. Y.S., J.W., J.H., S.L. and Y.L. carried
773 out experiments. J.H, S.L. and Y.L. performed data analysis. X.D., Y.S, J.W., and J.H.
774 wrote the paper.

775 **Ethics declarations**

776 Competing interests

777 The authors declare no competing interests.

778 **References**

- 779 Alfano, J. R., & Collmer, A. (1997). The type III (Hrp) secretion pathway of plant
780 pathogenic bacteria: trafficking harpins, Avr proteins, and death. *Journal of*
781 *bacteriology*, 179(18), 5655-5662.
- 782 Alon, U. (2007). Network motifs: theory and experimental approaches. *Nature*
783 *Reviews Genetics*, 8(6), 450-461.
- 784 Amaral, L. A. N., Scala, A., Barthelemy, M., & Stanley, H. E. (2000). Classes of
785 small-world networks. *Proceedings of the national academy of sciences*,
786 97(21), 11149-11152.
- 787 Badis, G., Berger, M. F., Philippakis, A. A., Talukder, S., Gehrke, A. R., Jaeger, S.
788 A., . . . Chen, X. (2009). Diversity and complexity in DNA recognition by
789 transcription factors. *Science*, 324(5935), 1720-1723.
- 790 Bailey, T. L., Boden, M., Buske, F. A., Frith, M., Grant, C. E., Clementi, L., . . . Noble,
791 W. S. (2009). MEME SUITE: tools for motif discovery and searching. *Nucleic*
792 *Acids Research*, 37(suppl_2), W202-W208.
- 793 Bender, C. L., Alarcón-Chaidez, F., & Gross, D. C. (1999). *Pseudomonas syringae*
794 phytotoxins: mode of action, regulation, and biosynthesis by peptide and
795 polyketide synthetases. *Microbiology and molecular biology reviews*, 63(2),
796 266-292.
- 797 Bhardwaj, N., Yan, K.-K., & Gerstein, M. B. (2010). Analysis of diverse regulatory
798 networks in a hierarchical context shows consistent tendencies for
799 collaboration in the middle levels. *Proceedings of the national academy of*

- 800 *sciences*, 107(15), 6841-6846.
- 801 Blasco, B., Chen, J. M., Hartkoorn, R., Sala, C., Uplekar, S., Rougemont, J., . . . Cole,
802 S. T. (2012). Virulence regulator EspR of *Mycobacterium tuberculosis* is a
803 nucleoid-associated protein. *PLoS Pathogens*, 8(3), e1002621.
- 804 Buell, C. R., Joardar, V., Lindeberg, M., Selengut, J., Paulsen, I. T., Gwinn, M. L., . . .
805 Kolonay, J. F. (2003). The complete genome sequence of the *Arabidopsis* and
806 tomato pathogen *Pseudomonas syringae* pv. *tomato* DC3000. *Proceedings of*
807 *the national academy of sciences*, 100(18), 10181-10186.
- 808 Burda, Z., Krzywicki, A., Martin, O. C., & Zagorski, M. (2011). Motifs emerge from
809 function in model gene regulatory networks. *Proceedings of the national*
810 *academy of sciences*, 108(42), 17263-17268.
- 811 Caillet, J., Baron, B., Boni, I. V., Caillet-Saguy, C., & Hajnsdorf, E. (2019).
812 Identification of protein-protein and ribonucleoprotein complexes containing
813 Hfq. *Sci Rep*, 9(1), 14054. doi:10.1038/s41598-019-50562-w
- 814 Chan, S. S.-K., & Kyba, M. (2013). What is a master regulator? *Journal of stem cell*
815 *research & therapy*, 3.
- 816 Chatterjee, A., Cui, Y., Yang, H., Collmer, A., Alfano, J. R., & Chatterjee, A. K.
817 (2003). GacA, the response regulator of a two-component system, acts as a
818 master regulator in *Pseudomonas syringae* pv. *tomato* DC3000 by controlling
819 regulatory RNA, transcriptional activators, and alternate sigma factors.
820 *Molecular Plant-Microbe Interactions*, 16(12), 1106-1117.
- 821 Cunnac, S., Lindeberg, M., & Collmer, A. (2009). *Pseudomonas syringae* type III

- 822 secretion system effectors: repertoires in search of functions. *Current opinion*
823 *in microbiology*, 12(1), 53-60.
- 824 Deng, X., Lan, L., Xiao, Y., Kennelly, M., Zhou, J.-M., & Tang, X. (2010).
825 *Pseudomonas syringae* two-component response regulator RhpR regulates
826 promoters carrying an inverted repeat element. *Molecular Plant-Microbe*
827 *Interactions*, 23(7), 927-939.
- 828 Deng, X., Liang, H., Chen, K., He, C., Lan, L., & Tang, X. (2014). Molecular
829 mechanisms of two-component system RhpRS regulating type III secretion
830 system in *Pseudomonas syringae*. *Nucleic Acids Research*, 42(18),
831 11472-11486.
- 832 Deng, X., Xiao, Y., Lan, L., Zhou, J.-M., & Tang, X. (2009). *Pseudomonas syringae*
833 pv. *phaseolicola* mutants compromised for type III secretion system gene
834 induction. *Molecular Plant-Microbe Interactions*, 22(8), 964-976.
- 835 Dittrich, M. T., Klau, G. W., Rosenwald, A., Dandekar, T., & Müller, T. (2008).
836 Identifying functional modules in protein–protein interaction networks: an
837 integrated exact approach. *Bioinformatics*, 24(13), i223-i231.
- 838 Fan, L., Wang, T., Hua, C., Sun, W., Li, X., Grunwald, L., . . . Yin, Y. (2020). A
839 compendium of DNA-binding specificities of transcription factors in
840 *Pseudomonas syringae*. *Nature Communications*, 11(1), 4947.
- 841 Fishman, M. R., Zhang, J., Bronstein, P. A., Stodghill, P., & Filiatrault, M. J. (2018).
842 Ca²⁺-induced two-component system CvsSR regulates the type III secretion
843 system and the extracytoplasmic function sigma factor AlgU in *Pseudomonas*

- 844 *syringae* pv. *tomato* DC3000. *Journal of bacteriology*, 200(5), 10.1128/jb.
845 00538-00517.
- 846 Fortuna, A., Collalto, D., Schiaffi, V., Pastore, V., Visca, P., Ascenzioni, F., . . . Leoni,
847 L. (2022). The *Pseudomonas aeruginosa* DksA1 protein is involved in H₂O₂
848 tolerance and within-macrophages survival and can be replaced by DksA2.
849 *Scientific Reports*, 12(1), 10404.
- 850 Galardini, M., Brilli, M., Spini, G., Rossi, M., Roncaglia, B., Bani, A., . . . Bacci, G.
851 (2015). Evolution of intra-specific regulatory networks in a multipartite
852 bacterial genome. *PLoS Computational Biology*, 11(9), e1004478.
- 853 Gerstein, M. B., Kundaje, A., Hariharan, M., Landt, S. G., Yan, K.-K., Cheng, C., . . .
854 Alexander, R. (2012). Architecture of the human regulatory network derived
855 from ENCODE data. *Nature*, 489(7414), 91-100.
- 856 Goldbeter, A., & Koshland Jr, D. E. (1984). Ultrasensitivity in biochemical systems
857 controlled by covalent modification. Interplay between zero-order and
858 multistep effects. *Journal of Biological Chemistry*, 259(23), 14441-14447.
- 859 He, S. Y. (1998). Type III protein secretion systems in plant and animal pathogenic
860 bacteria. *Annual review of phytopathology*, 36(1), 363-392.
- 861 Hendrickson, E. L., Guevera, P., & Ausubel, F. M. (2000). The alternative sigma
862 factor RpoN is required for *hrp* activity in *Pseudomonas syringae* pv.
863 *maculicola* and acts at the level of *hrpL* transcription. *Journal of bacteriology*,
864 182(12), 3508-3516.
- 865 Hirano, S. S., & Upper, C. D. (2000). Bacteria in the leaf ecosystem with emphasis on

- 866 *Pseudomonas syringae*—a pathogen, ice nucleus, and epiphyte. *Microbiology*
867 *and molecular biology reviews*, 64(3), 624-653.
- 868 Hua, C., Huang, J., Wang, T., Sun, Y., Liu, J., Huang, L., & Deng, X. (2022). Bacterial
869 transcription factors bind to coding regions and regulate internal cryptic
870 promoters. *MBio*, 13(5), e01643-01622.
- 871 Hua, C., Wang, T., Shao, X., Xie, Y., Huang, H., Liu, J., . . . Jiang, L. (2020).
872 *Pseudomonas syringae* dual-function protein Lon switches between virulence
873 and metabolism by acting as both DNA-binding transcriptional regulator and
874 protease in different environments. *Environmental Microbiology*, 22(7),
875 2968-2988.
- 876 Huang, J., Yao, C., Sun, Y., Ji, Q., & Deng, X. (2022). Virulence-related regulatory
877 network of *Pseudomonas syringae*. *Computational and Structural*
878 *Biotechnology Journal*.
- 879 Ichinose, Y., Taguchi, F., & Mukaihara, T. (2013). Pathogenicity and virulence factors
880 of *Pseudomonas syringae*. *Journal of general plant pathology*, 79, 285-296.
- 881 Ishihama, A., Shimada, T., & Yamazaki, Y. (2016). Transcription profile of
882 *Escherichia coli*: genomic SELEX search for regulatory targets of
883 transcription factors. *Nucleic Acids Research*, 44(5), 2058-2074.
- 884 Jeong, H., Tombor, B., Albert, R., Oltvai, Z. N., & Barabási, A.-L. (2000). The
885 large-scale organization of metabolic networks. *Nature*, 407(6804), 651-654.
- 886 Jolma, A., Yan, J., Whittington, T., Toivonen, J., Nitta, K. R., Rastas, P., . . . Wei, G.
887 (2013). DNA-binding specificities of human transcription factors. *Cell*, 152(1),

- 888 327-339.
- 889 Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., . . .
890 Hassabis, D. (2021). Highly accurate protein structure prediction with
891 AlphaFold. *nature*, 596(7873), 583-589. doi:10.1038/s41586-021-03819-2
- 892 Kawakita, Y., Taguchi, F., Inagaki, Y., Toyoda, K., Shiraishi, T., & Ichinose, Y. (2012).
893 Characterization of each *aeiR* and *mexT* mutant in *Pseudomonas syringae* pv.
894 *tabaci* 6605. *Molecular genetics and genomics*, 287, 473-484.
- 895 King, E. O., Ward, M. K., & Raney, D. E. (1954). Two simple media for the
896 demonstration of pyocyanin and fluorescin. *The Journal of laboratory and*
897 *clinical medicine*, 44(2), 301-307.
- 898 Kvitko, B. H., & Collmer, A. (2011). Construction of *Pseudomonas syringae* pv.
899 *tomato* DC3000 mutant and polymutant strains. *Plant immunity: methods and*
900 *protocols*, 109-128.
- 901 Lambert, S. A., Jolma, A., Campitelli, L. F., Das, P. K., Yin, Y., Albu, M., . . .
902 Weirauch, M. T. (2018). The human transcription factors. *Cell*, 172(4),
903 650-665.
- 904 Lan, L., Deng, X., Zhou, J., & Tang, X. (2006). Genome-wide gene expression
905 analysis of *Pseudomonas syringae* pv. *tomato* DC3000 reveals overlapping
906 and distinct pathways regulated by *hrpL* and *hrpRS*. *Molecular Plant-Microbe*
907 *Interactions*, 19(9), 976-987.
- 908 Lee, T. I., Rinaldi, N. J., Robert, F., Odom, D. T., Bar-Joseph, Z., Gerber, G. K., . . .
909 Simon, I. (2002). Transcriptional regulatory networks in *Saccharomyces*

- 910 *cerevisiae*. *Science*, 298(5594), 799-804.
- 911 Lee, T. I., & Young, R. A. (2013). Transcriptional regulation and its misregulation in
912 disease. *Cell*, 152(6), 1237-1251.
- 913 Ma, H.-W., Kumar, B., Ditges, U., Gunzer, F., Buer, J., & Zeng, A.-P. (2004). An
914 extended transcriptional regulatory network of *Escherichia coli* and analysis
915 of its hierarchical structure and network motifs. *Nucleic Acids Research*,
916 32(22), 6643-6649.
- 917 Mathelier, A., Shi, W., & Wasserman, W. W. (2015). Identification of altered
918 cis-regulatory elements in human disease. *Trends in Genetics*, 31(2), 67-76.
- 919 Meddows, T. R., Savory, A. P., Grove, J. I., Moore, T., & Lloyd, R. G. (2005). RecN
920 protein and transcription factor DksA combine to promote faithful
921 recombinational repair of DNA double-strand breaks. *Molecular microbiology*,
922 57(1), 97-110.
- 923 Milo, R., Shen-Orr, S., Itzkovitz, S., Kashtan, N., Chklovskii, D., & Alon, U. (2002).
924 Network motifs: simple building blocks of complex networks. *Science*,
925 298(5594), 824-827.
- 926 Newman, M. E. (2001). The structure of scientific collaboration networks.
927 *Proceedings of the national academy of sciences*, 98(2), 404-409.
- 928 Olesen, J. M., Bascompte, J., Dupont, Y. L., & Jordano, P. (2007). The modularity of
929 pollination networks. *Proceedings of the national academy of sciences*,
930 104(50), 19891-19896.
- 931 Papavassiliou, K. A., & Papavassiliou, A. G. (2016). Transcription factor drug targets.

- 932 *Journal of cellular biochemistry*, 117(12), 2693-2696.
- 933 Perez, J. C., & Groisman, E. A. (2009). Evolution of transcriptional regulatory circuits
934 in bacteria. *Cell*, 138(2), 233-244.
- 935 Rico, A., McCraw, S. L., & Preston, G. M. (2011). The metabolic interface between
936 *Pseudomonas syringae* and plant cells. *Current opinion in microbiology*, 14(1),
937 31-38.
- 938 Sawada, T., Eguchi, M., Asaki, S., Kashiwagi, R., Shimomura, K., Taguchi, F., . . .
939 Toyoda, K. (2018). MexEF-OprN multidrug efflux pump transporter
940 negatively controls N-acyl-homoserine lactone accumulation in *Pseudomonas*
941 *syringae* pv. *tabaci* 6605. *Molecular genetics and genomics*, 293, 907-917.
- 942 Shao, X., Tan, M., Xie, Y., Yao, C., Wang, T., Huang, H., . . . Han, L. (2021).
943 Integrated regulatory network in *Pseudomonas syringae* reveals dynamics of
944 virulence. *Cell Reports*, 34(13).
- 945 Shao, X., Xie, Y., Zhang, Y., & Deng, X. (2019). Biofilm formation assay in
946 *Pseudomonas syringae*. *Bio-protocol*, 9(10), e3237-e3237.
- 947 Shen-Orr, S. S., Milo, R., Mangan, S., & Alon, U. (2002). Network motifs in the
948 transcriptional regulation network of *Escherichia coli*. *Nature genetics*, 31(1),
949 64-68.
- 950 Simon, I., Barnett, J., Hannett, N., Harbison, C. T., Rinaldi, N. J., Volkert, T. L., . . .
951 Jaakkola, T. S. (2001). Serial regulation of transcriptional regulators in the
952 yeast cell cycle. *Cell*, 106(6), 697-708.
- 953 Strogatz, S. H. (2001). Exploring complex networks. *Nature*, 410(6825), 268-276.

- 954 Sun, Y., Shao, X., Zhang, Y., Han, L., Huang, J., Xie, Y., . . . Deng, X. (2022).
955 Maintenance of tRNA and elongation factors supports T3SS proteins
956 translational elongations in pathogenic bacteria during nutrient starvation. *Cell*
957 & *Bioscience*, 12(1), 1-17.
- 958 Taguchi, F., & Ichinose, Y. (2011). Role of type IV pili in virulence of *Pseudomonas*
959 *syringae* pv. *tabaci* 6605: correlation of motility, multidrug resistance, and
960 HR-inducing activity on a nonhost plant. *Molecular Plant-Microbe*
961 *Interactions*, 24(9), 1001-1011.
- 962 Teixeira, M. C., Monteiro, P. T., Palma, M., Costa, C., Godinho, C. P., Pais, P., . . .
963 Pedreira, T. (2018). YEASTRACT: an upgraded database for the analysis of
964 transcription regulatory networks in *Saccharomyces cerevisiae*. *Nucleic Acids*
965 *Research*, 46(D1), D348-D353.
- 966 Thieffry, D., Huerta, A. M., Pérez-Rueda, E., & Collado-Vides, J. (1998). From
967 specific gene regulation to genomic networks: a global analysis of
968 transcriptional regulation in *Escherichia coli*. *Bioessays*, 20(5), 433-440.
- 969 Tian, Z.-X., Fargier, E., Mac Aogain, M., Adams, C., Wang, Y.-P., & O’Gara, F.
970 (2009). Transcriptome profiling defines a novel regulon modulated by the
971 LysR-type transcriptional regulator MexT in *Pseudomonas aeruginosa*.
972 *Nucleic Acids Research*, 37(22), 7546-7559.
- 973 Trouillon, J., Imbert, L., Villard, A.-M., Vernet, T., Attrée, I., & Elsen, S. (2021).
974 Determination of the two-component systems regulatory network reveals core
975 and accessory regulations across *Pseudomonas aeruginosa* lineages. *Nucleic*

- 976 *Acids Research*, 49(20), 11476-11490.
- 977 van Duin, L., Krautz, R., Rennie, S., & Andersson, R. (2023). Transcription factor
978 expression is the main determinant of variability in gene co-activity.
979 *Molecular Systems Biology*, e11392.
- 980 Wade, J. T. (2015). Mapping transcription regulatory networks with ChIP-seq and
981 RNA-seq. *Prokaryotic systems biology*, 119-134.
- 982 Wang, J., Shao, X., Zhang, Y., Zhu, Y., Yang, P., Yuan, J., . . . Chen, S. (2018). HrpS is
983 a global regulator on type III secretion system (T3SS) and non-T3SS genes in
984 *Pseudomonas savastanoi* pv. *phaseolicola*. *Molecular Plant-Microbe*
985 *Interactions*, 31(12), 1232-1243.
- 986 Wang, J., Zhuang, J., Iyer, S., Lin, X., Whitfield, T. W., Greven, M. C., . . . Cheng, Y.
987 (2012). Sequence features and chromatin structure around the genomic regions
988 bound by 119 human transcription factors. *Genome research*, 22(9),
989 1798-1812.
- 990 Whitchurch, C. B., Tolker-Nielsen, T., Ragas, P. C., & Mattick, J. S. (2002).
991 Extracellular DNA required for bacterial biofilm formation. *Science*,
992 295(5559), 1487-1487.
- 993 Wilson, D., Charoensawan, V., Kummerfeld, S. K., & Teichmann, S. A. (2008).
994 DBD-taxonomically broad transcription factor predictions: new content and
995 functionality. *Nucleic Acids Research*, 36(suppl_1), D88-D92.
- 996 Winsor, G. L., Griffiths, E. J., Lo, R., Dhillon, B. K., Shay, J. A., & Brinkman, F. S.
997 (2016). Enhanced annotations and features for comparing thousands of

- 998 *Pseudomonas* genomes in the *Pseudomonas* genome database. *Nucleic Acids*
999 *Research*, 44(D1), D646-D653.
- 1000 Xiao, Y., & Hutcheson, S. W. (1994). A single promoter sequence recognized by a
1001 newly identified alternate sigma factor directs expression of pathogenicity and
1002 host range determinants in *Pseudomonas syringae*. *Journal of bacteriology*,
1003 176(10), 3089-3091.
- 1004 Xiao, Y., Lan, L., Yin, C., Deng, X., Baker, D., Zhou, J.-M., & Tang, X. (2007).
1005 Two-component sensor RhpS promotes induction of *Pseudomonas syringae*
1006 type III secretion system by repressing negative regulator RhpR. *Molecular*
1007 *Plant-Microbe Interactions*, 20(3), 223-234.
- 1008 Xie, Y., Ding, Y., Shao, X., Yao, C., Li, J., Liu, J., & Deng, X. (2021). *Pseudomonas*
1009 *syringae* senses polyphenols via phosphorelay crosstalk to inhibit virulence.
1010 *EMBO reports*, 22(12), e52805.
- 1011 Xie, Y., Li, J., Ding, Y., Shao, X., Sun, Y., Xie, F., . . . Deng, X. (2022). An atlas of
1012 bacterial two-component systems reveals function and plasticity in signal
1013 transduction. *Cell Reports*, 41(3).
- 1014 Xie, Y., Shao, X., & Deng, X. (2019). Regulation of type III secretion system in
1015 *Pseudomonas syringae*. *Environmental Microbiology*, 21(12), 4465-4477.
- 1016 Yan, Q., Rogan, C. J., Pang, Y.-Y., Davis, E. W., & Anderson, J. C. (2020). Ancient
1017 co-option of an amino acid ABC transporter locus in *Pseudomonas syringae*
1018 for host signal-dependent virulence gene regulation. *PLoS Pathogens*, 16(7),
1019 e1008680.

1020 Zhang, Y., Liu, T., Meyer, C. A., Eeckhoute, J., Johnson, D. S., Bernstein, B. E., . . . Li,
1021 W. (2008). Model-based analysis of ChIP-Seq (MACS). *Genome biology*, *9*(9),
1022 1-9.

1023 Zhu, C., Byers, K. J., McCord, R. P., Shi, Z., Berger, M. F., Newburger, D. E., . . .
1024 Radhakrishnan, M. (2009). High-resolution DNA-binding specificity analysis
1025 of yeast transcription factors. *Genome research*, *19*(4), 556-566.

1026 Zhu, L. J., Gazin, C., Lawson, N. D., Pagès, H., Lin, S. M., Lapointe, D. S., & Green,
1027 M. R. (2010). ChIPpeakAnno: a Bioconductor package to annotate ChIP-seq
1028 and ChIP-chip data. *BMC bioinformatics*, *11*, 1-10.

1029

1030 **Figure legends**

1031 **Figure 1. Hierarchical height and collaboration of TFs reveal the multiple**
1032 **regulatory patterns in *P. syringae*.**

1033 **a**, Close-up representation of 262 TFs hierarchy in *P. syringae*. Nodes depict TFs.
1034 Colors of edges represent source-bases. **b**, Enrichment of different collaborating
1035 (direct interaction, indirect interaction and cooperativity) TF-pairs at top (T), middle
1036 (M) and bottom (B) levels. Gray nodes below the graph represent TFs. The dashed
1037 orange line indicates the averaged level of collaboration. **c**, Thirteen Three-node
1038 sub-modules with the number of occurrences and an example. Spire loop is the most
1039 enriched sub-module. Edges represent the regulatory direction. **d**, Autoregulations are
1040 accompanied by the number of occurrences and 13 auto-regulators as examples.

1041 **Figure 2. Bottom TFs share the same binding sequences to coregulate in *P.***
1042 ***syringae*.**

1043 **a**, The co-association map for 170 TFs in *P. syringae* shows the co-associated scores
1044 of binding peaks of these TFs (rows) that overlap each TF peak (columns). The three
1045 colored rectangles represent three different TF levels. C1-C4 represent four clusters of
1046 TFs according to the co-associated scores. The TFs in corresponding cluster are
1047 shown in the circle diagram. Orange nodes represent top TFs. Green nodes represent
1048 middle TFs. Purple nodes represent bottom TFs. The colors of edges are the mixture
1049 of two source TF colors. **b**, The heat-map of MexT indicates the associated scores of
1050 binding peaks of TFs in C3 (columns) that overlap the binding peaks (rows) of MexT.

1051 PSPPH2411, PSPPH3643 and CysI are the top 3 TFs with high associated scores. **c**,
1052 UpSet plot shows the number of genes uniquely targeted TFs or co-targeted by
1053 multiple TFs in C3. The vertical black lines represent shared TF-binding sites. The y
1054 axis represents the number of overlapped binding sites across the linked TFs. The x
1055 axis represents the number of binding sites for each TF. Orange line represents the
1056 most enriched gene *cysI* that is co-targeted by 12 TFs in C3. **d**, Motifs of MexT and
1057 other 10 TFs in C3 which show similar binding sequences.

1058 **Figure 3. Virulence hierarchical regulatory network reveals 35 TFs involved in**
1059 **virulence.**

1060 **a**, Virulence hierarchical regulatory network shows the TF hierarchy and the large
1061 pool of target genes of multi-TF. Target genes are related with seven key virulence
1062 pathways, including biofilm formation, secondary messengers, motility, T3SS, QS,
1063 phytotoxin production and siderophore transporter. Orange nodes represent top TFs.
1064 Green nodes represent middle TFs. Purple nodes represent bottom TFs. Blue nodes
1065 represent target genes. Yellow edges represent downward point. Red edges represent
1066 upward point. **b**, The head-to-head binding motif of PSPPH1951, the validation of the
1067 binding sites of PSPPH1951 by EMSA, and the detection of expression of target gene
1068 *hopAE1* in WT, Δ PSPPH1951 and complementary strain by RT-qPCR. The validated
1069 binding sites are from the promoters of the *hrpR*, *hopAE1* and *hopAH2*. **c**, The
1070 binding motif of PSPPH2193 is head-to-head. EMSA confirms the direct binding of
1071 PSPPH2193 to the promoters of *fleQ* and *flhF*. RT-qPCR confirms the positive
1072 regulation of PSPPH2193 on the expression of *fleQ* and *flhF*. Motility assay validates

1073 the weaker motility of Δ PSPPH2193 than WT and complementary strain. **d**, The
1074 binding motif of PSPPH3268 is head-to-head. EMSA confirms the direct binding of
1075 PSPPH3268 to the promoters of *hrpR*, *alg44* and *pilM*. RT-qPCR confirms the
1076 negative regulation of PSPPH3268 on the expression of *alg44*, *algX* and *pilM*. Crystal
1077 violet staining assay and the quantification of biofilm formation validate the negative
1078 regulation of PSPPH3268 on the biofilm formation. Congo red assay confirms the
1079 negative regulation of PSPPH3268 on colony morphologies and EPS production.
1080 Student's *t* test. n.s., not significant, * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$.

1081 **Figure 4. Hundreds of TFs are identified to participate in metabolic pathways.**

1082 **a**, Metabolic hierarchical regulatory network shows the TF hierarchy and the large
1083 pool of target genes of multi-TF. Target genes are related with eight key metabolic
1084 pathways, including biosynthesis of amino acids, DNA replication, ABC transporter,
1085 oxidative phosphorylation, TCA cycle, RNA polymerase, phosphonate metabolism
1086 and methyl-accepting chemotaxis. Orange nodes represent top TFs. Green nodes
1087 represent middle TFs. Purple nodes represent bottom TFs. Blue nodes represent target
1088 genes. Yellow edges represent the direct interaction. **b**, Radar plots show the putative
1089 key regulators identified in six different metabolic pathways, including TCA cycle,
1090 oxidative phosphorylation, methyl-accepting, biosynthesis of amino acids, RNA
1091 polymerase and ABC transporter. Each radiation line represents a key regulator, and
1092 the radial length of the thick colored line is the rate of target genes to the associated
1093 genes, representing the significance of the enrichment of the TF target genes within
1094 each pathway. **c**, TFs involved in the methyl-accepting pathway bound to the

1095 promoters of TFs in the same category. Brown nodes represent the TFs that are
1096 responsible for methyl-accepting pathway. The brown arrows point to the targeted TFs.
1097 **d**, TFs involved in the oxidative phosphorylation pathway bind to the promoters of
1098 TFs in the methyl-accepting pathway. Red nodes represent the TFs that are
1099 responsible for oxidative phosphorylation pathway. Brown nodes represent the TFs
1100 that are responsible for methyl-accepting pathway. Blue nodes represent the TFs
1101 involved in these two pathways. The arrows point to the targeted TFs and the arrow
1102 colors are source-based.

1103 **Figure 5. Various conservations are observed in TFs between different *P. syringae***
1104 **pathovars.**

1105 **a**, Proportion of the TF target genes detected in one, two, three or four tested genomes.
1106 c1-c4 represent the conservation of targets in one, two, three and four strains. **b-c**,
1107 Repartition of the total pool of target genes (**b**) or TF-target interactions (**c**) in four
1108 tested strains. **d**, Enrichment coverage tracks of ChIP-seq against negative controls for
1109 TF Irp with binding sites on the promoter of *gidA* in all four genomes. **e**, Enrichment
1110 coverage tracks of ChIP-seq against negative controls (gray tracks) for the three TFs
1111 (Irp, PSPPH2193 and PSPPH4127) with binding sites on the promoter of *rpoD* in four
1112 tested strains.

1113 **Figure 6. Functional modularized regulatory network in *P. syringae* exhibits the**
1114 **specific functions of both TFs and their target genes.**

1115 **a**, The functional categorical regulation network in *P. syringae* analyzed by Gephi

1116 (resolution 0.9). The 16 modules (both TFs and their target genes) are labeled in
1117 different colors. TF nodes are shown as corresponding sized circles representing their
1118 expression level. Their target genes are shown as corresponding-colored dots in the
1119 background. TF-target edges are shown as corresponding-colored lines between nodes.
1120 **b**, Graph diagram indicates the connections between TF and their targets in modules.
1121 Module nodes are shown as corresponding-colored circles with size proportional to
1122 the number of nodes within. Edge colors are source-based, and edge thicknesses
1123 represent the connected quantity between modules. **c-d**, Functional category
1124 enrichment analysis of genes in each module.

1125 **Figure 7. Global transcriptional regulatory network in *P. syringae*.**

1126 The integrated transcriptional regulatory network reflects the interactions between all
1127 TFs classified into 39 families from different DNA binding domain types and target
1128 genes annotated from pathway annotations. The targets are shown in 12 pathways
1129 with various colors. TF nodes are gray as corresponding sizes representing TF number
1130 in the family. Edge colors are target-based.

1131 **Figure S1. Summary of ChIP-seq results in *P. syringae*.**

1132 **a**, Locations of all the 301 annotated TFs in *P. syringae* genome. Blue lines represent
1133 the TF loci. **b**, ChIPed TFs are classified as respective family with different colors.
1134 Square size represents the targeted enrichment of each TF. **c**, The preferential
1135 enrichment at different genome loci of each ChIPed TF, including upstream,
1136 overlapStart, inside, overlapEnd, downstream and includeFeature regions.

1137 **Figure S2. Graph diagram of feed-forward loop in *P. syringae*.**

1138 TF columns in feed-forward loop (M13, n=696) are classified as families. TF-TF
1139 edges are distinguished with separate colors.

1140 **Figure S3. Co-association and virulence-related functional category of TFs at**
1141 **three different levels.**

1142 **a-b**, The co-associated map and functional category of top TFs. The peak number of
1143 top TFs are shown at upper corresponding to the TFs in the lower map. **c-d**, The
1144 co-associated map and functional category of middle TFs. **e-f**, The co-associated map
1145 and functional category of bottom TFs.

1146 **Figure S4. The validation of the binding sites of virulence-related TFs in *P.***
1147 ***syringae*.**

1148 **a-c**, The promoter of *rpoD* is the negative control of PSPPH1951 and PSPPH2193.
1149 The promoter of PSPPH3658 is the negative control of PSPPH3268. **d**, The validation
1150 of binding sites of PSPPH1951. The validated binding sites are from the promoters of
1151 *pilZ*, *pilF* and *pilG*. **e**, The validation of binding sites of PSPPH3798. The validated
1152 binding sites are from the promoters of *fliK*, *fliE*, *fliD* and *fleQ*. The promoter of
1153 PSPPH2263 is the negative control. **f**, A regulatory cascade of PSPPH3504. / means
1154 sibling nodes. – means downward regulation.

1155 **Figure S5. Metabolic functional category of TFs at three different levels.**

1156 **a-c**, Functional category according to different metabolic pathways of top (**a**), middle

1157 (b) and bottom (c) TFs.

1158 **Figure S6. Key TFs in different metabolic pathways.**

1159 **a**, Radar plots show the putative key regulators identified in two different metabolic
1160 pathways, including DNA replication, and phosphonate and phosphonate metabolism.

1161 Each radiation line represents a key regulator, and the radial length of the thick
1162 colored line is the rate of target genes to the associated genes, representing the

1163 significance of the enrichment of the TF target genes within each pathway. **b**, The

1164 monomer motif of PSPPH0755 and the validation of the binding sites of PSPPH0755

1165 by EMSA. The validated binding sites are from promoters of PSPPH5210 and

1166 PSPPH3109. The promoter of PSPPH4598 is negative control. **c**, The head-to-head

1167 motif of PSPPH3798 and the validation of the binding sites of PSPPH3798 by EMSA.

1168 The validated binding sites are from promoters of PSPPH3881 and PSPPH5119. **d**,

1169 The monomer motif of PSPPH4638 and the validation of the binding sites of

1170 PSPPH4638 by EMSA. The validated binding sites are from promoters of

1171 PSPPH3881 and PSPPH0550. The promoter of PSPPH4598 is the negative control.

Figure 1.

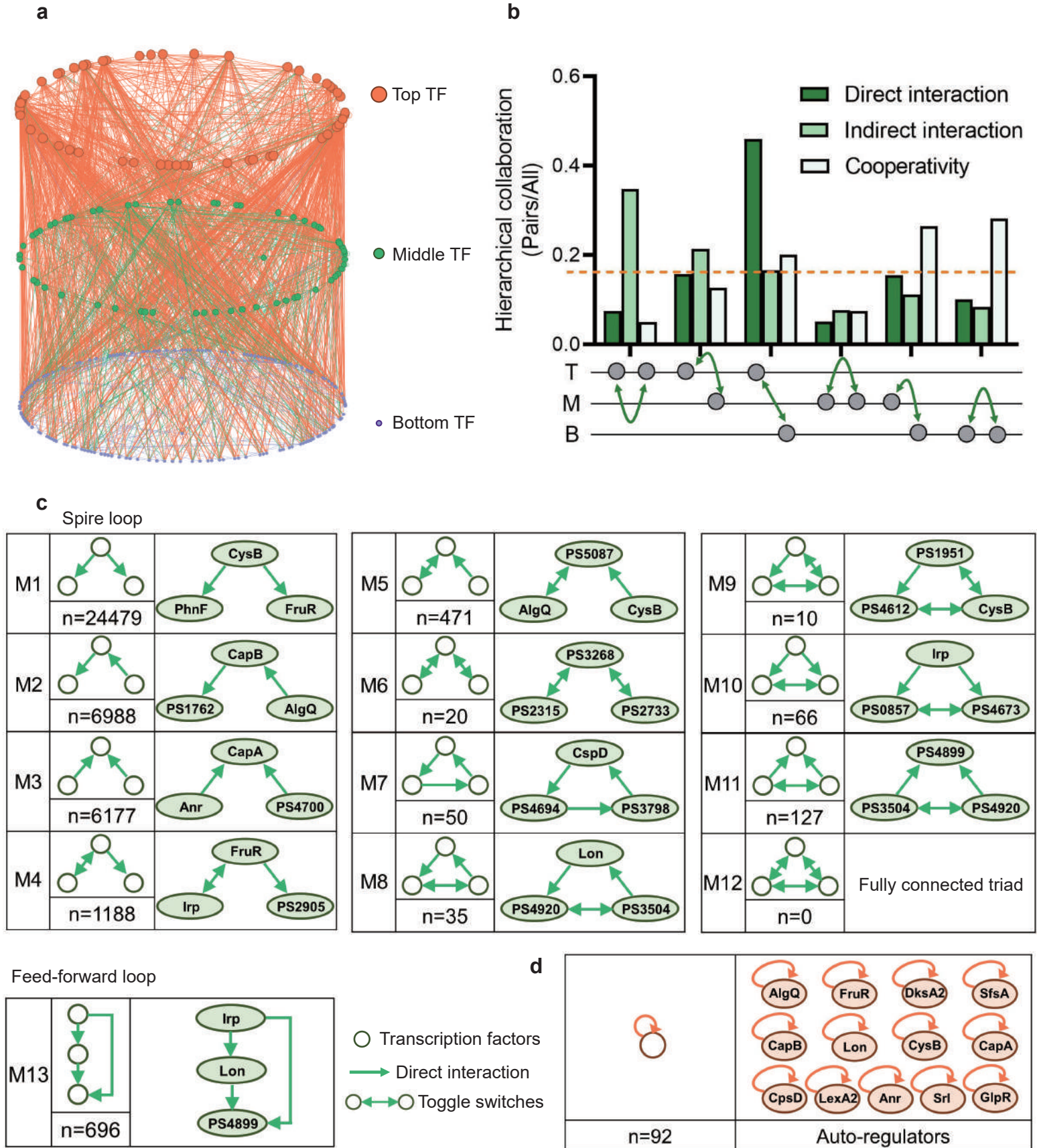
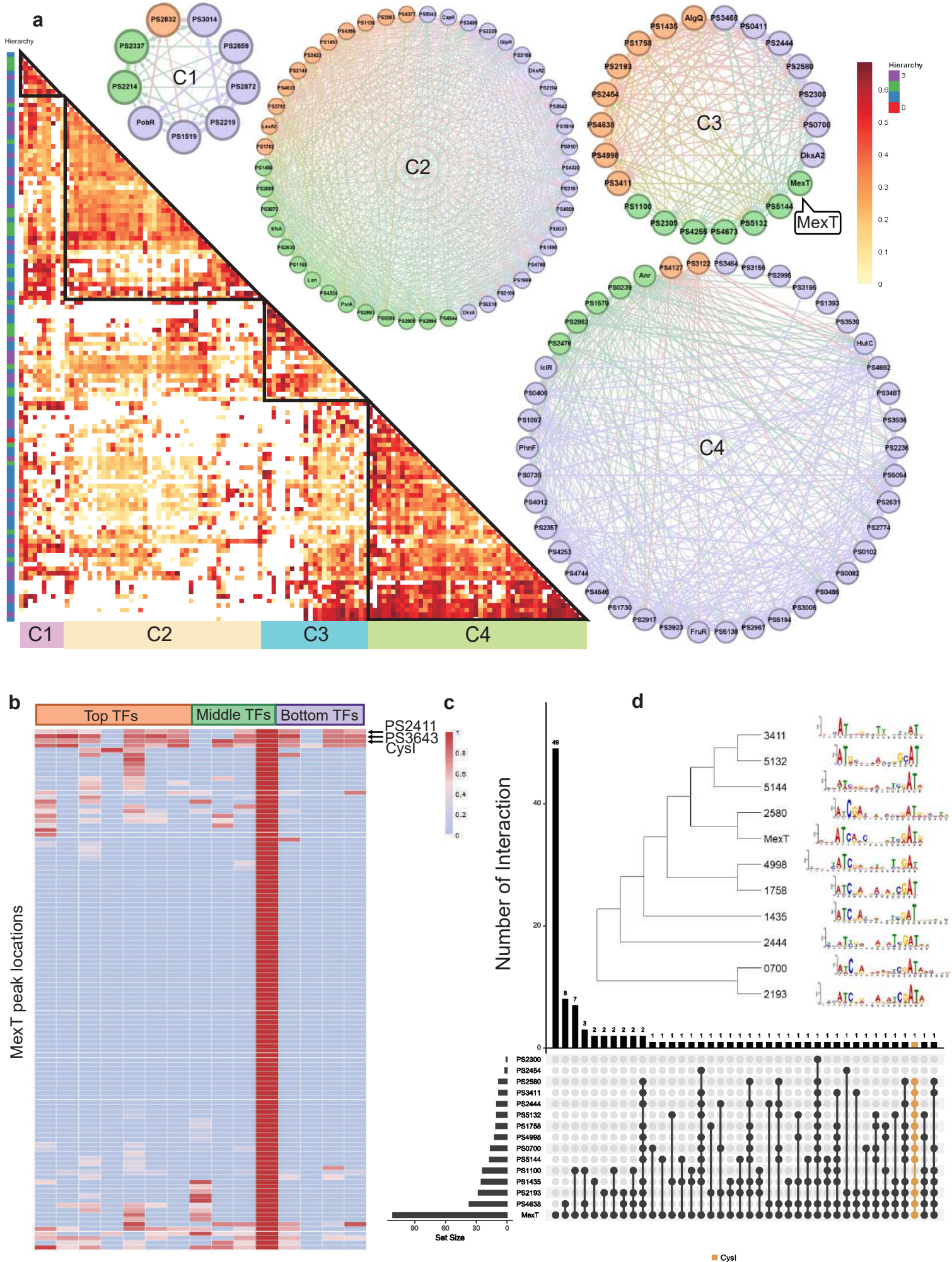


Figure 2.



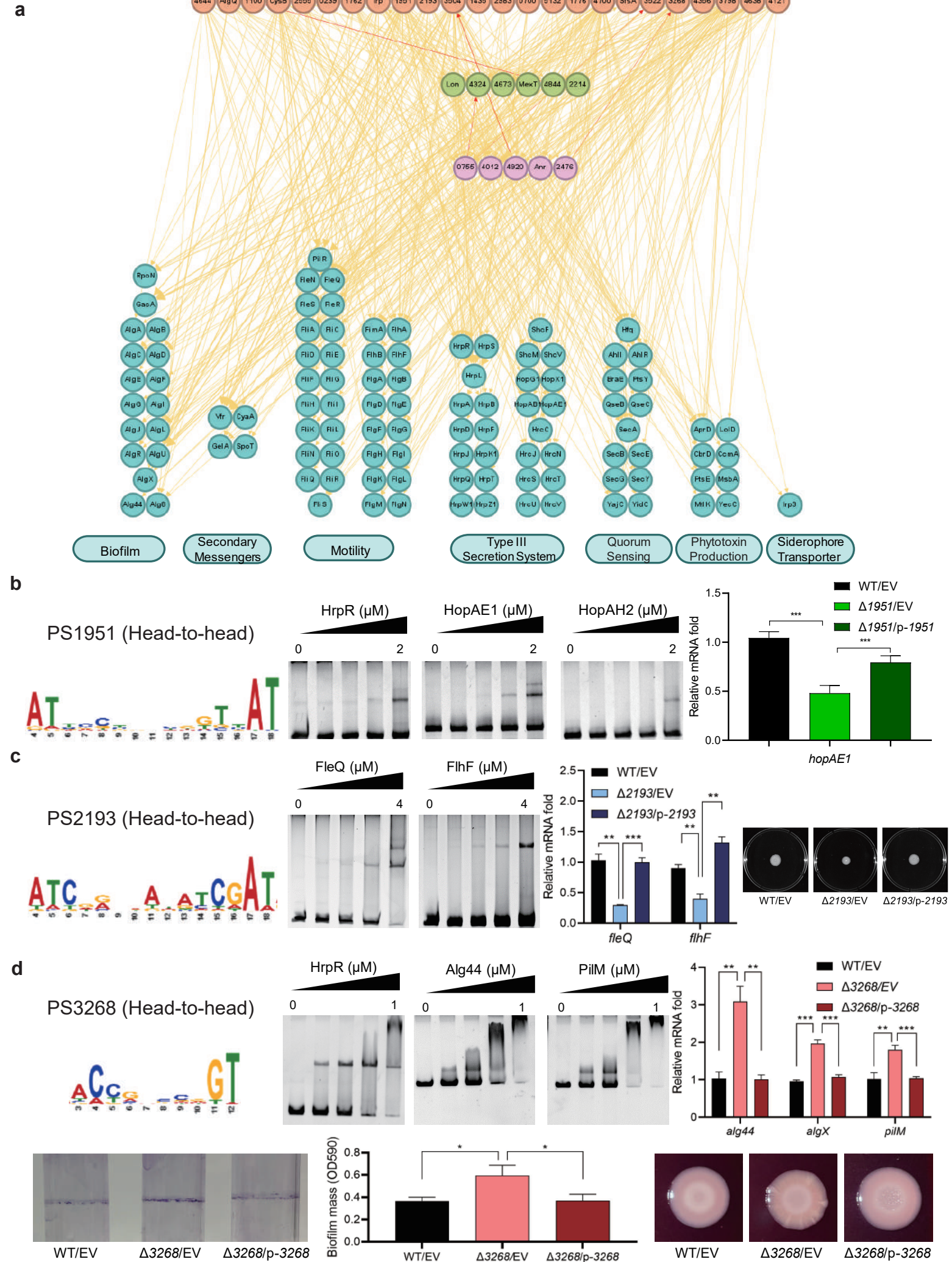
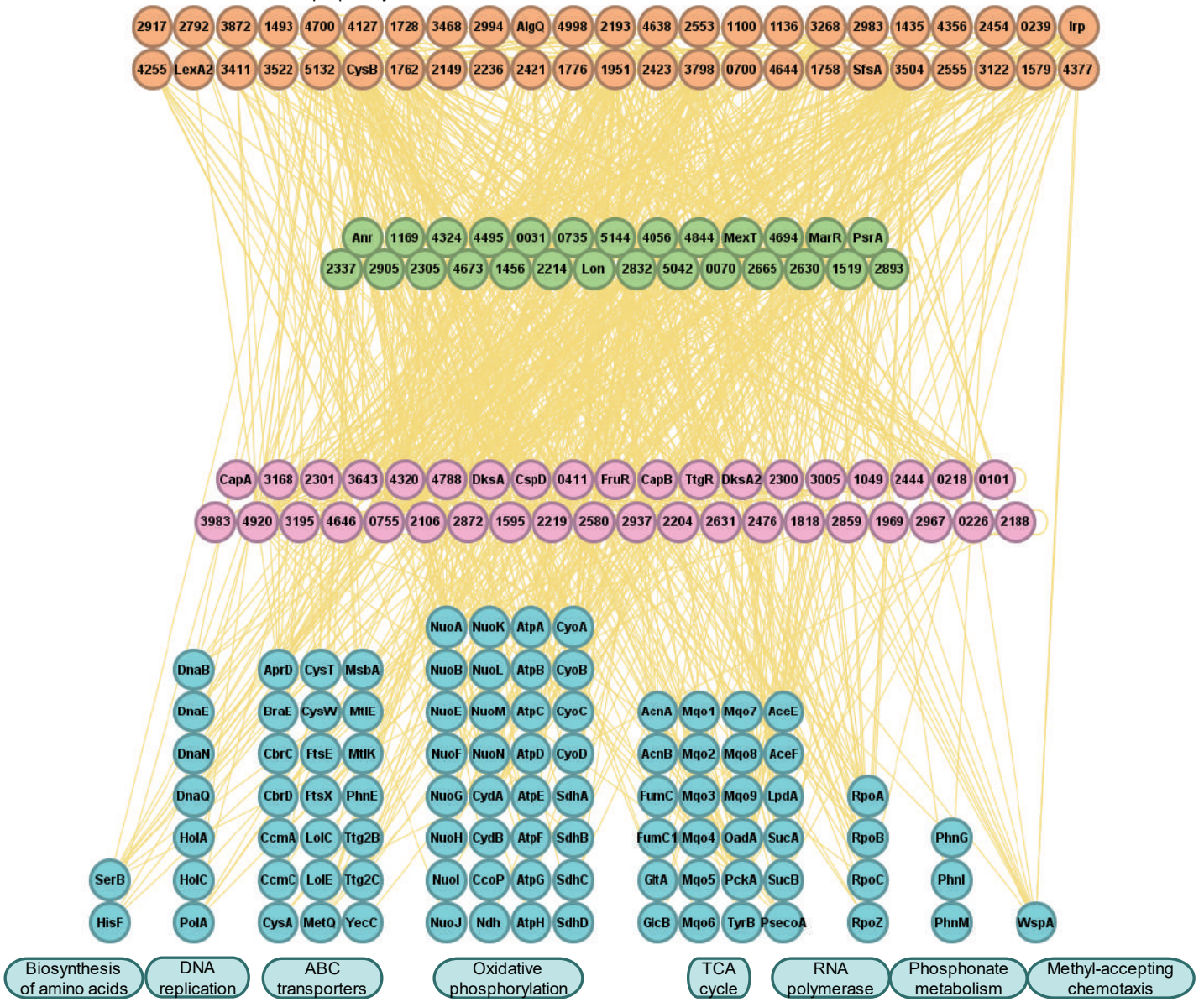
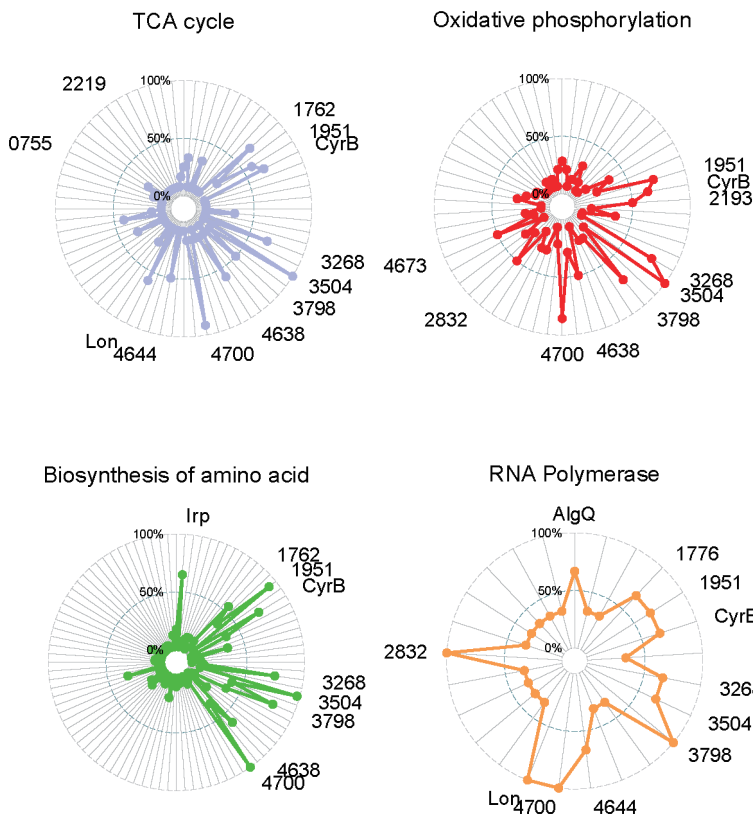


Figure 4.

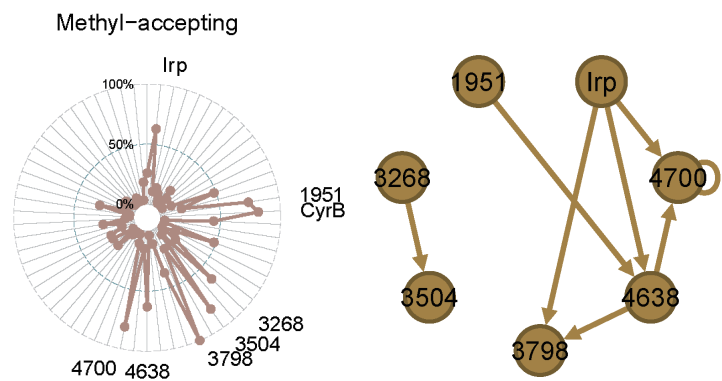
a



b



c



d

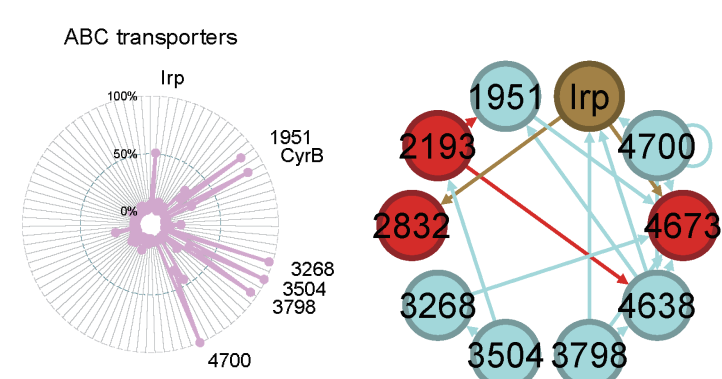


Figure 5.

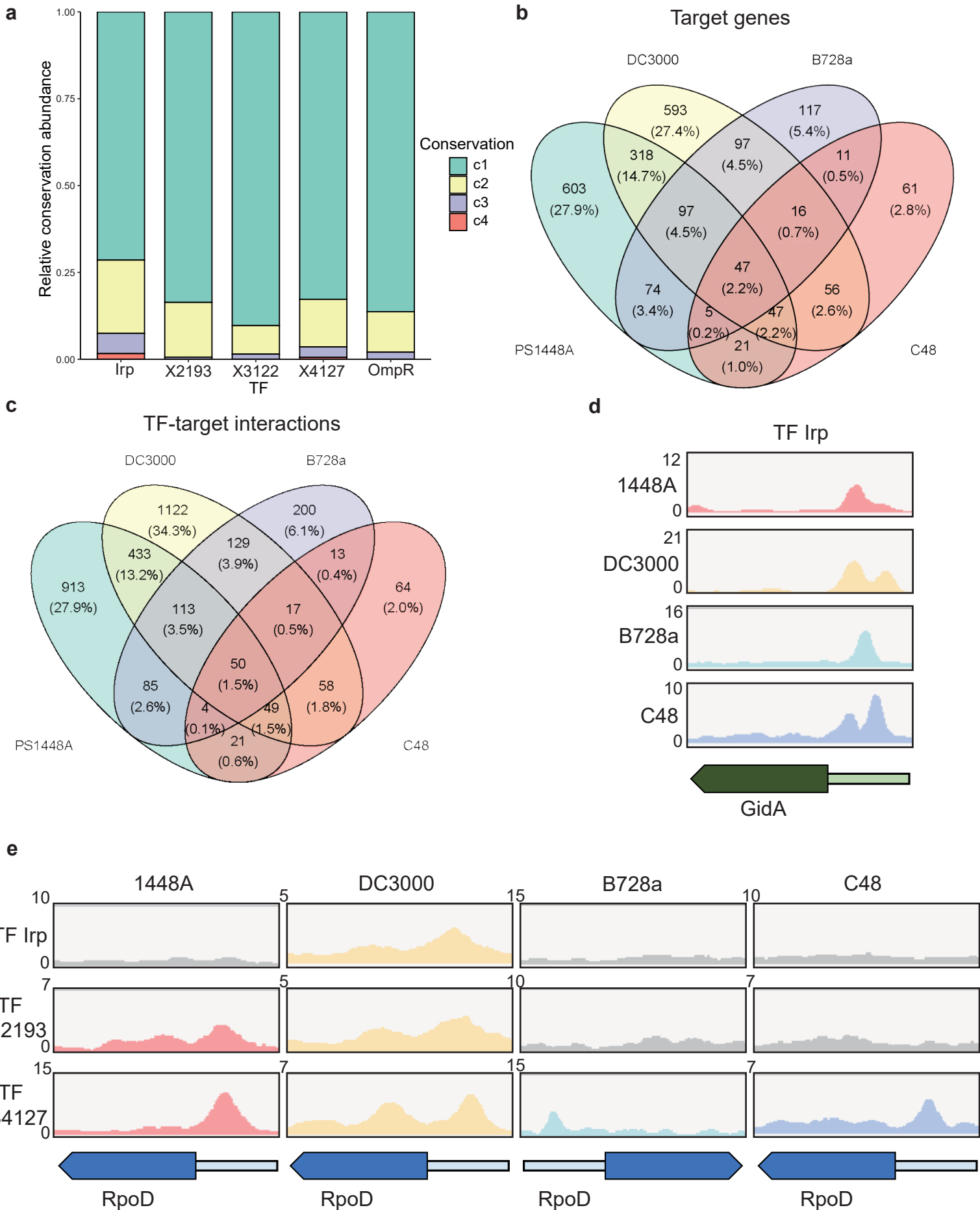
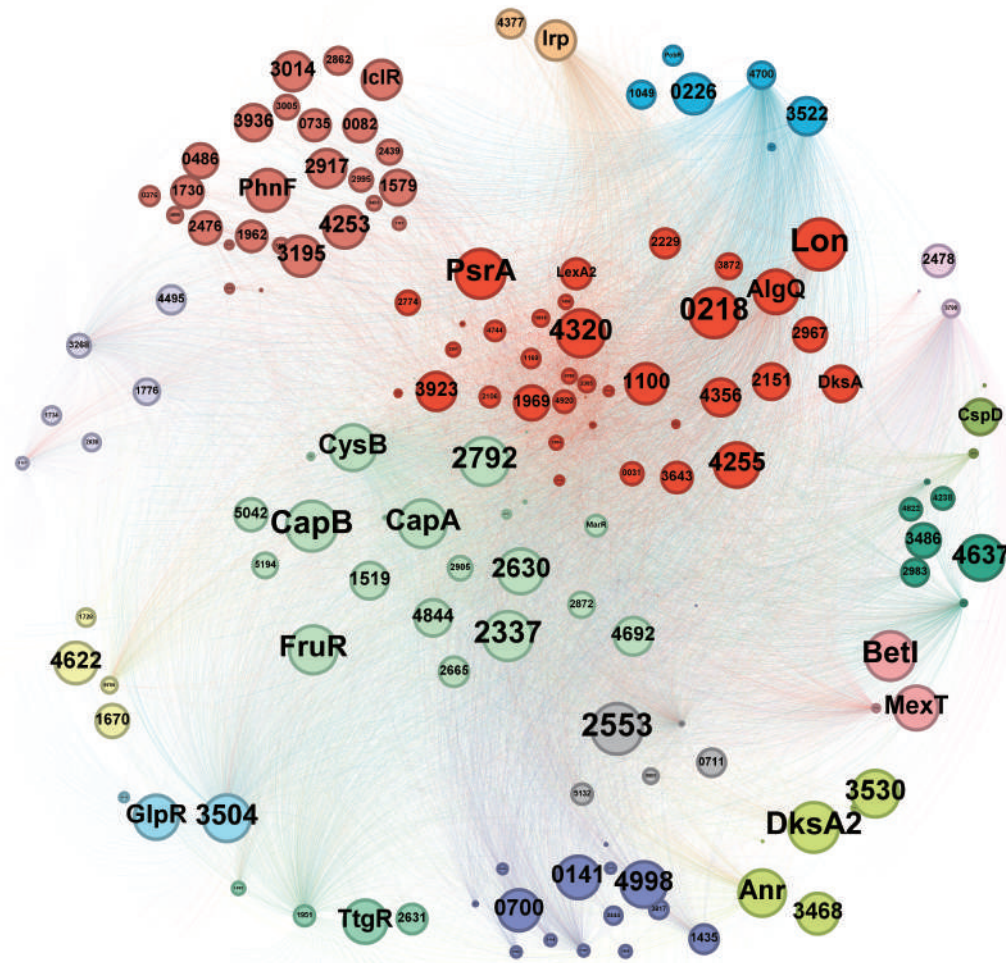
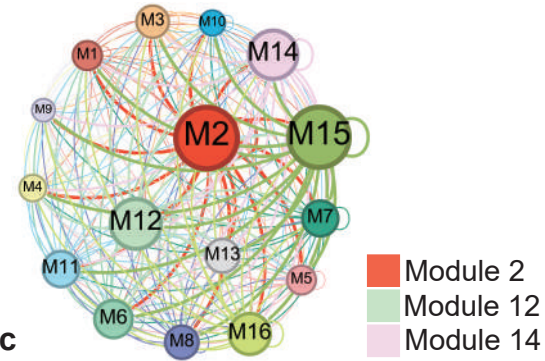


Figure 6.

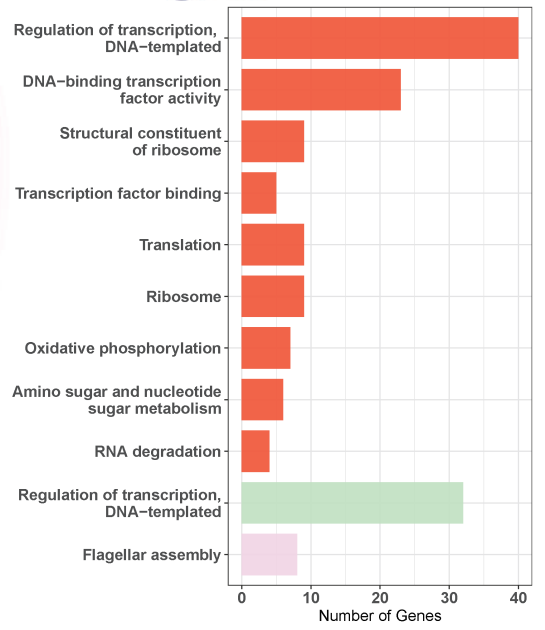
a



b



c



d

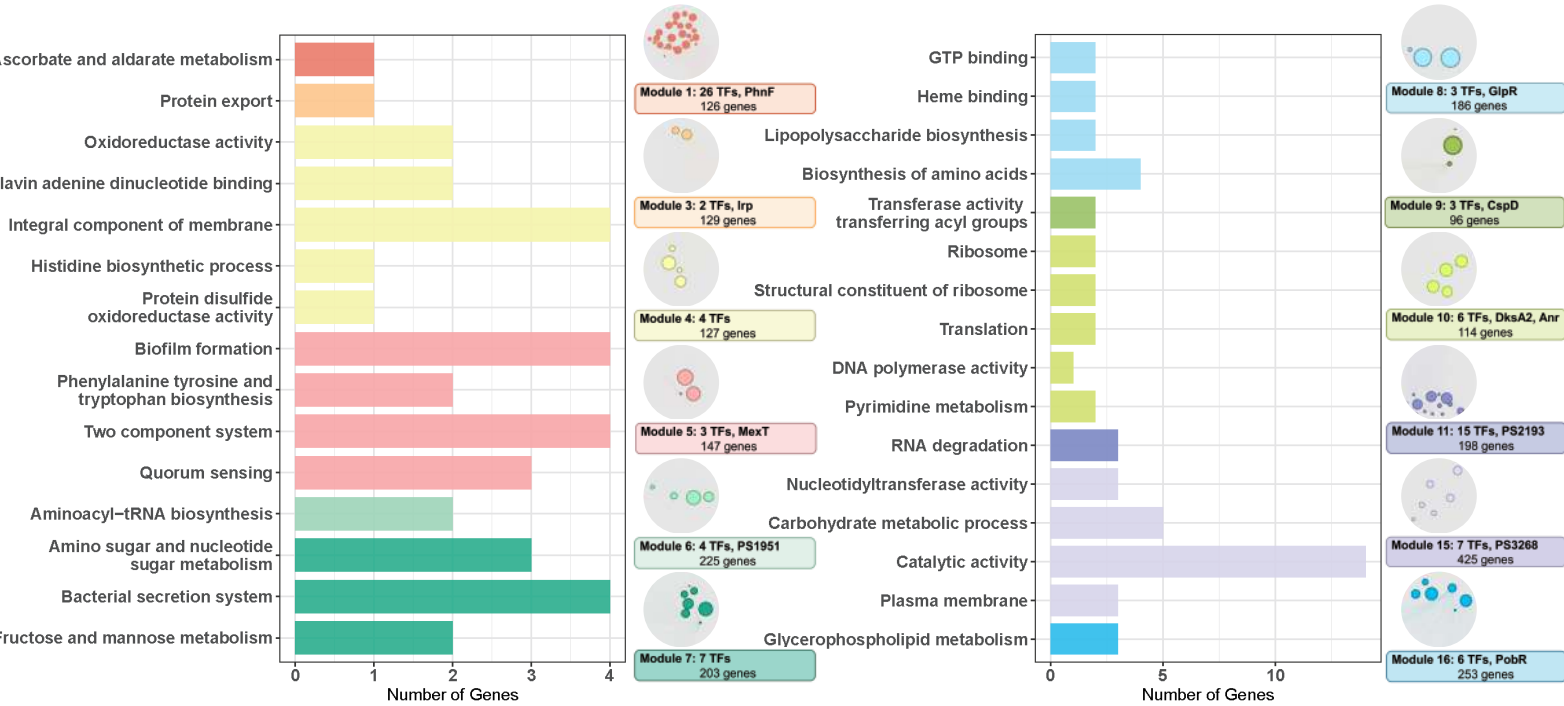


Figure 7.

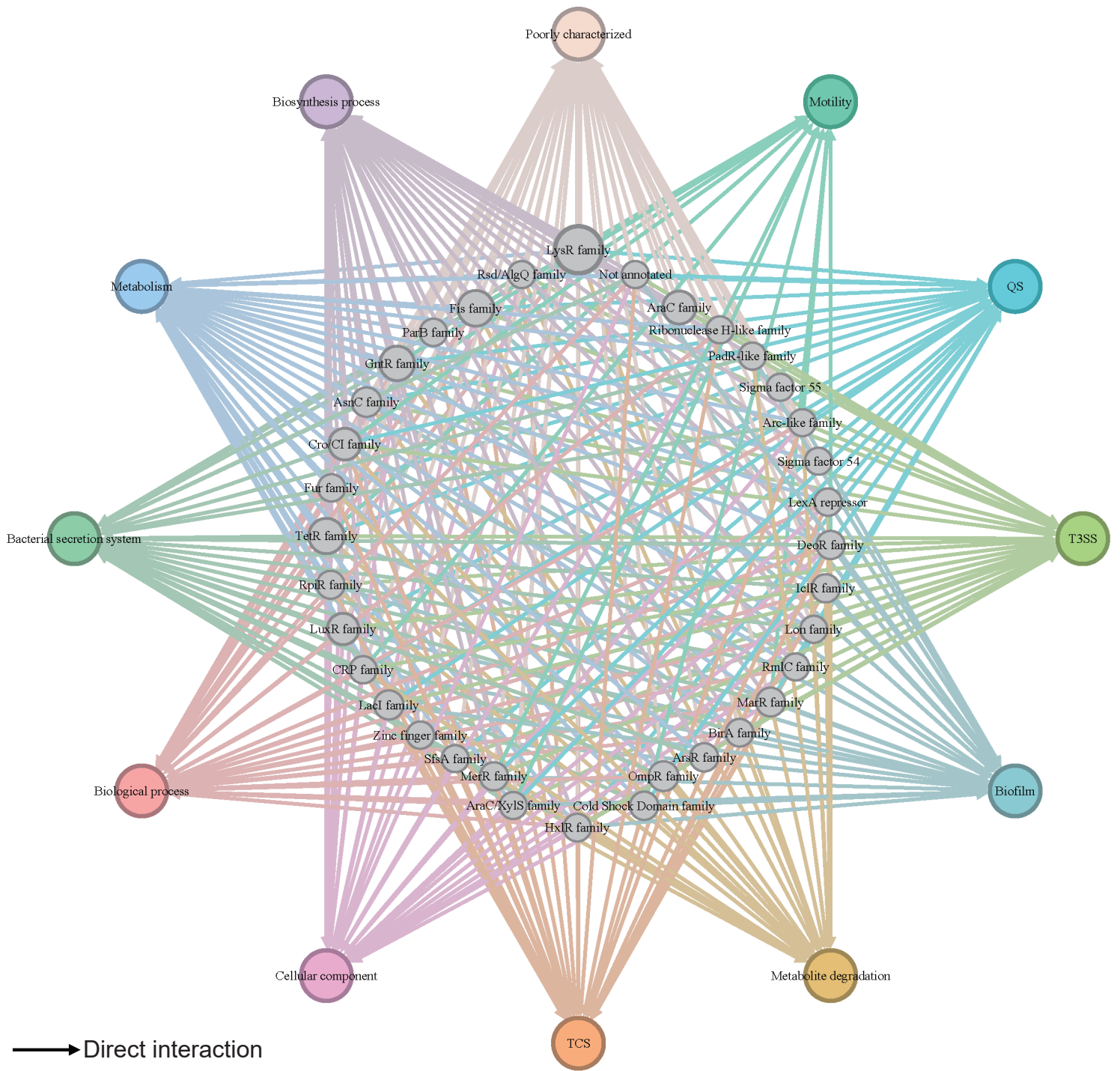
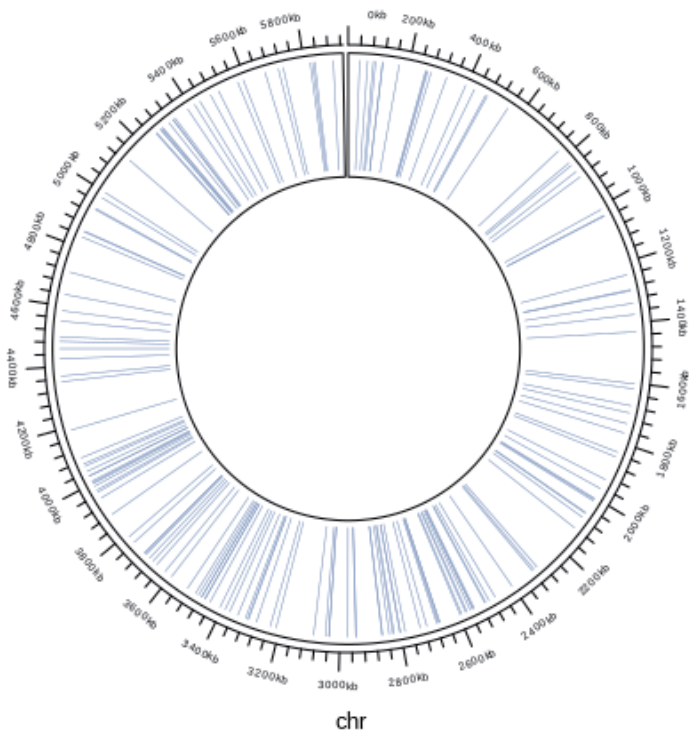
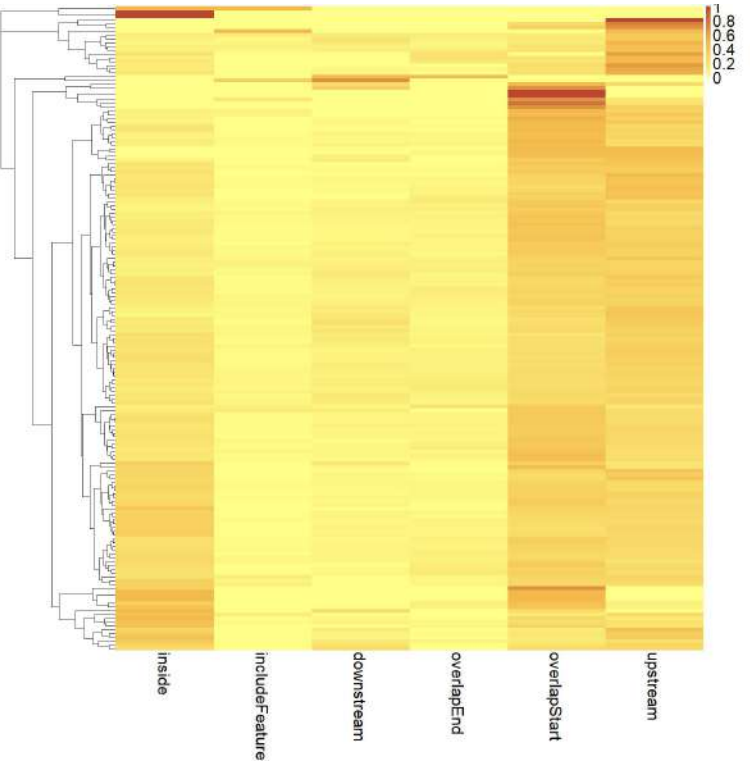


Figure S1

a



c



b

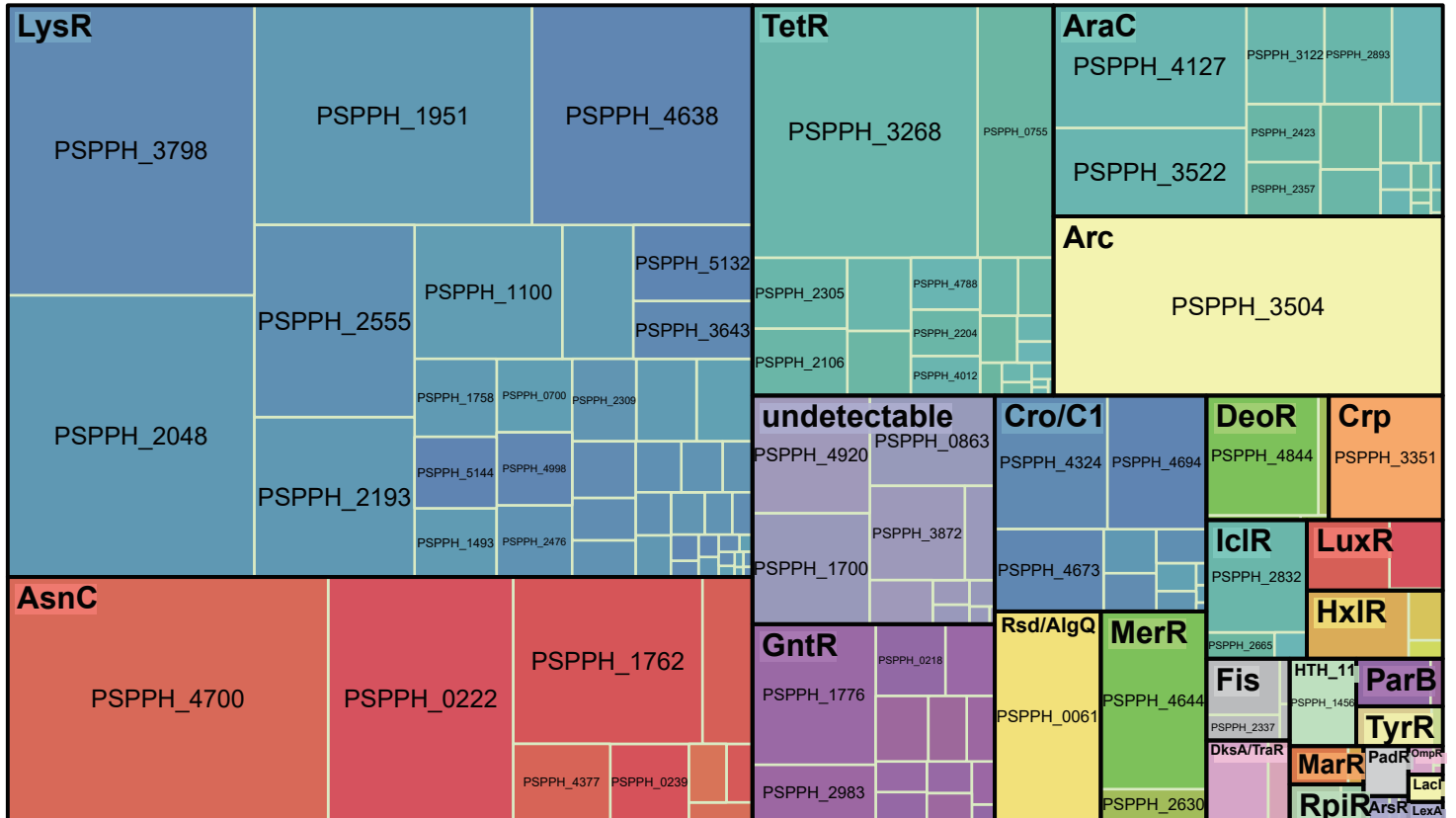


Figure S2

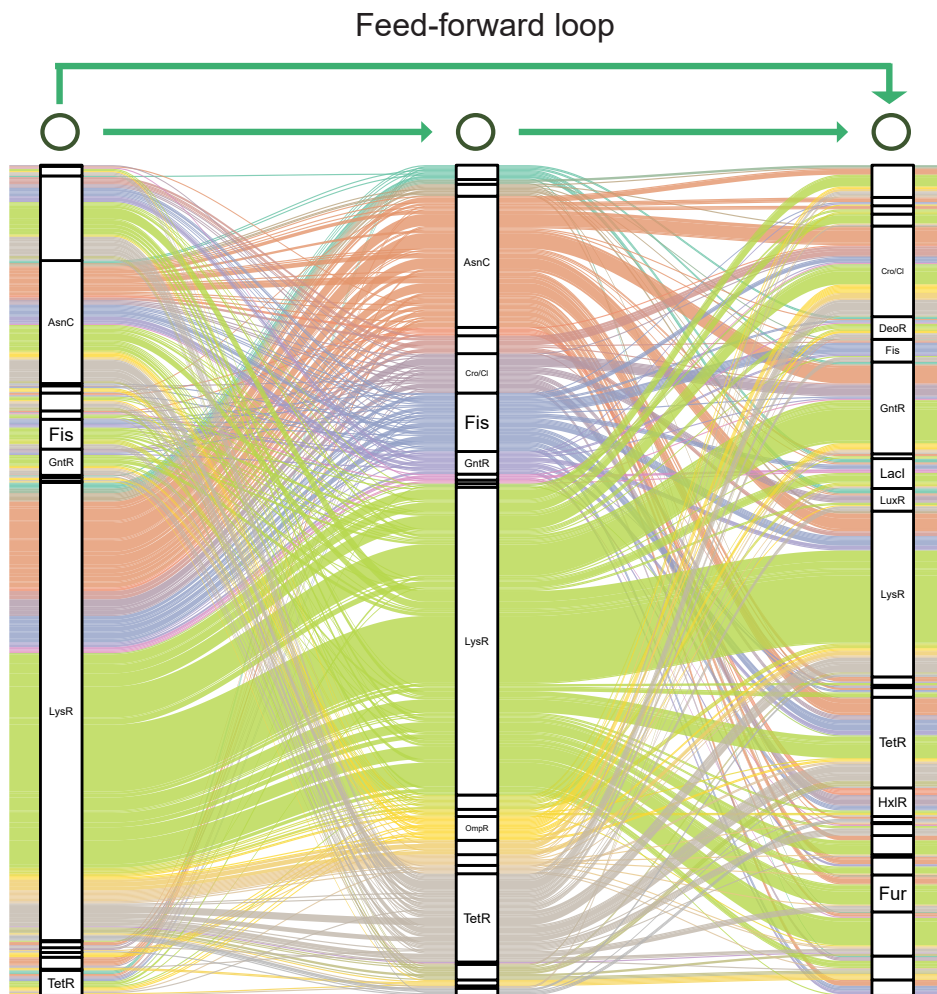


Figure S3.

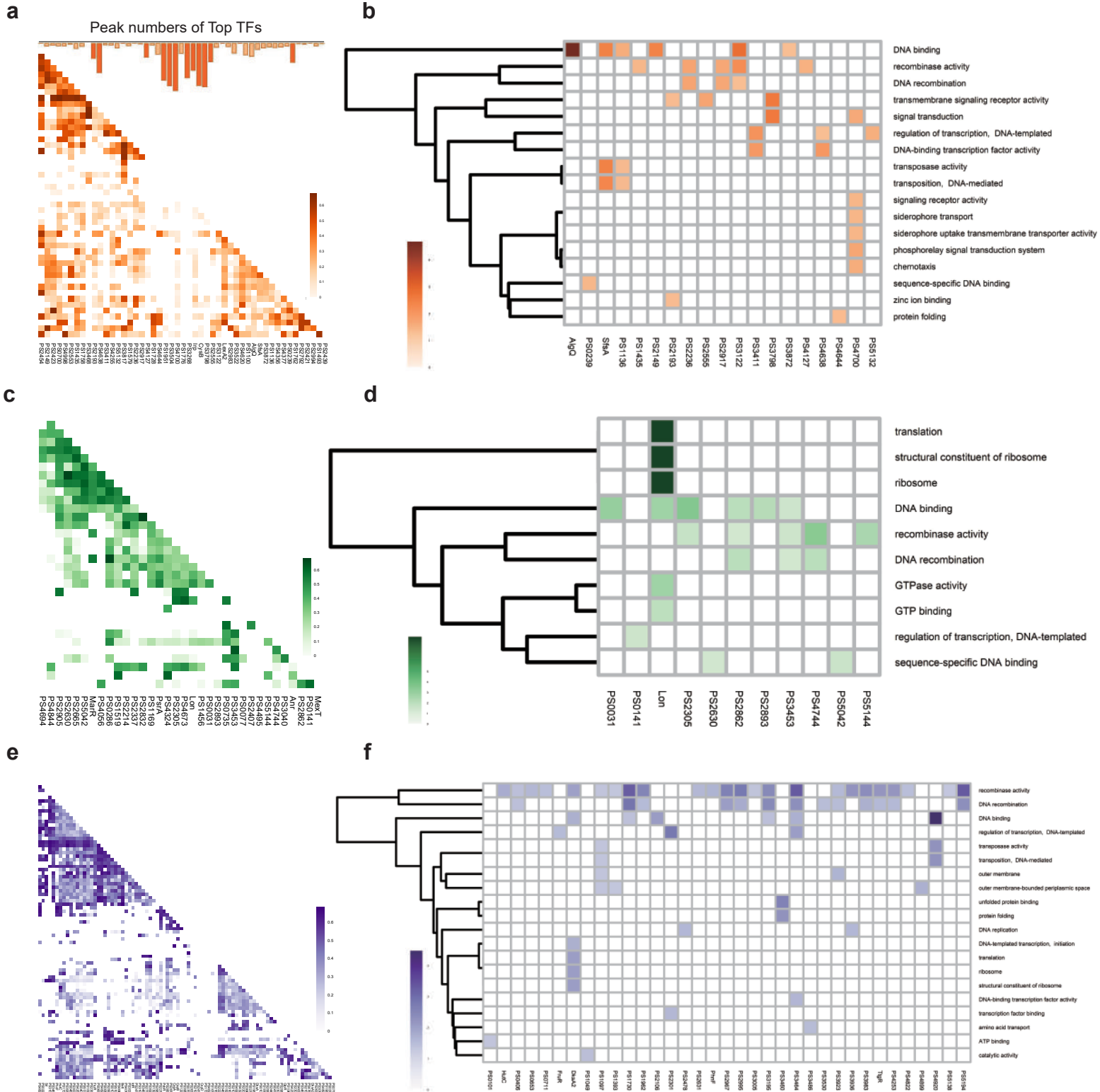


Figure S4

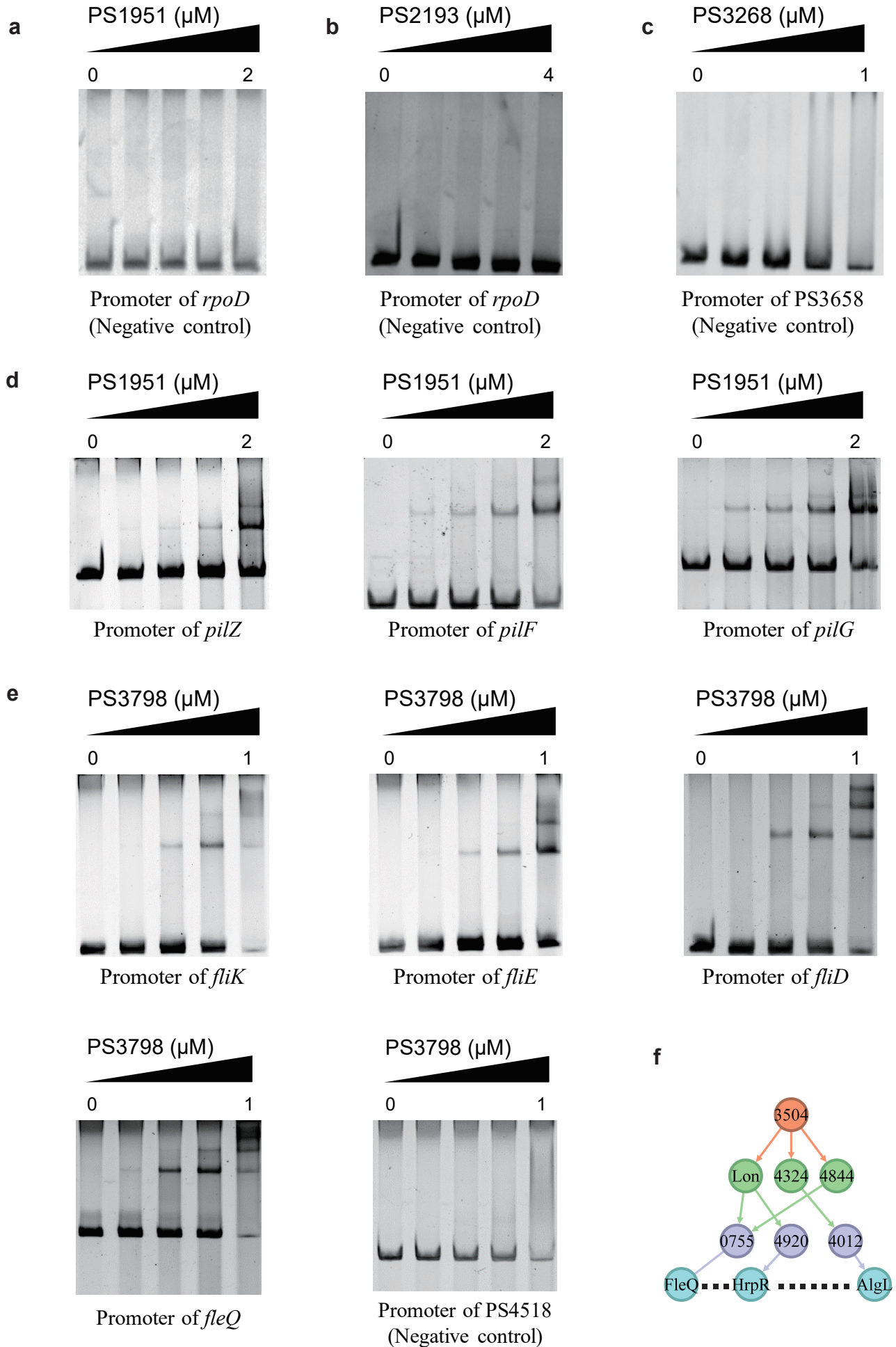


Figure S5.

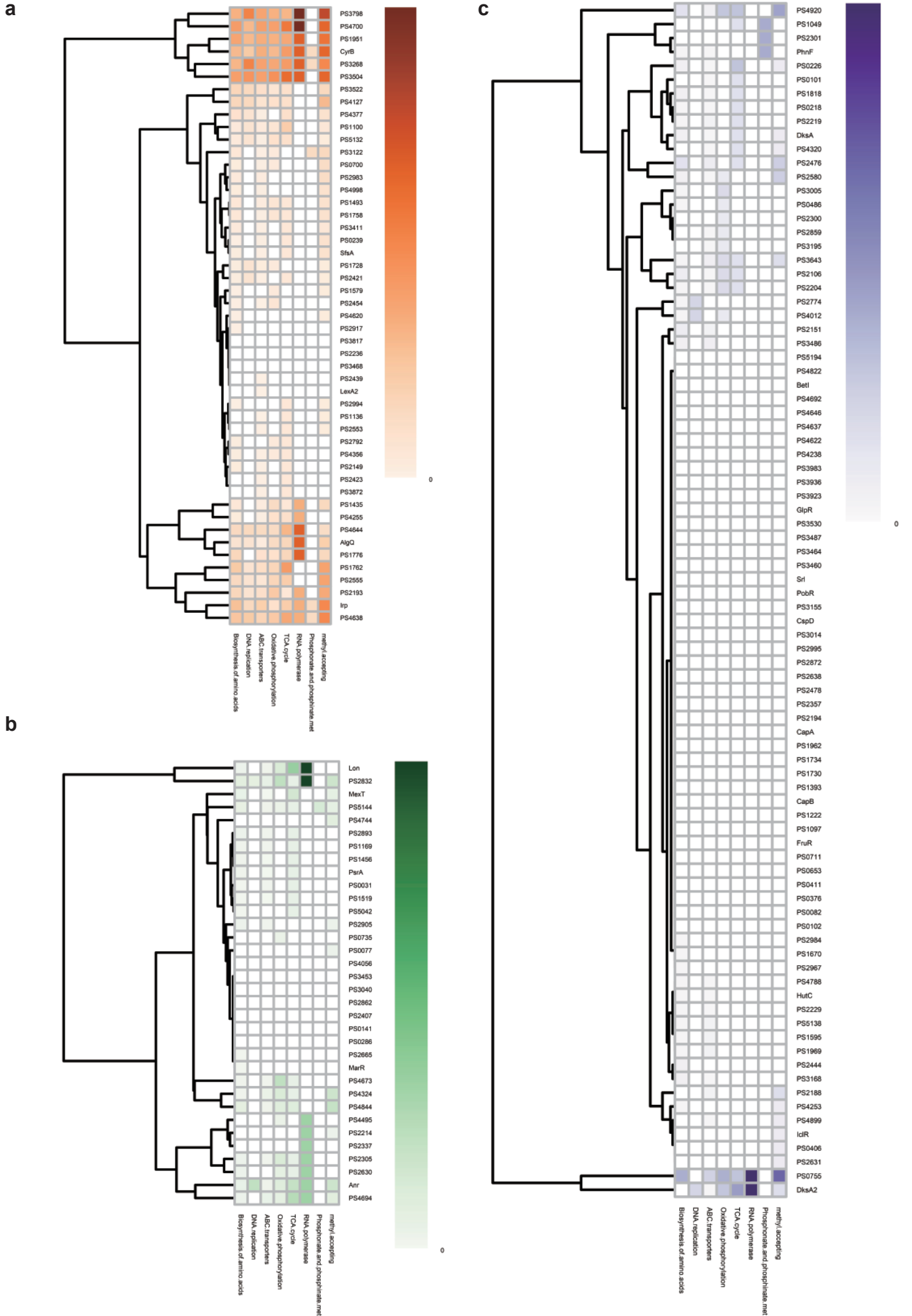


Figure S6

