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Additional Information

The ABCF3 gene of Arabidopsis is functionally linked with GCN1 but not with GCN2 during stress and development.

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18 **Key message:** The Arabidopsis ABCF3 gene of involved in developmental and
19 stress-related processes, working together with the GCN1 gene but independently
20 of the phosphorylation of the translation initiation factor 2

21 **Abstract**

22 One of the main mechanisms regulating translation is the one based on the
23 phosphorylation of the alpha subunit of the translation initiation factor 2 (eIF2α)
24 by the General Control Non-repressive 2 (GCN2) protein kinase. In yeast, this
25 kinase binds to two scaffold proteins (GCN1 and GCN20), facilitating its
26 activation on translating ribosomes. Homologous of the three proteins exist in
27 Arabidopsis. In this species, whereas the kinase is activated under several stress
28 situations, the involvement of the scaffold proteins in those processes is
29 controversial, and a new role for GCN1 in translation, independent of the
30 phosphorylation of eIF2α, has been proposed. Arabidopsis present five genes with
31 homology to GCN20 (ABCF1 to 5) in its genome. We show here that any of these
32 five genes is needed for eIF2α phosphorylation. Furthermore, plant phenotypes
33 under abiotic stresses and chloroplast development suggest that ABCF3 is
34 functionally link with GCN1, but not with GCN2. Finally, *gcn1* and *abcf3* mutants
35 share similar transcriptional reprogramming, affecting photosynthesis and stress
36 responses. The common down-regulation of regulators of the flagellin receptor
37 FLS2 in both mutants suggest that the observed defect in pathogen-associated
38 molecular pattern (PAMP)-induced stomatal closure of these two mutants could
39 be mediated by these proteins.

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44 **Keywords**: transcriptomic, chloroplast, translation, defense

45 **Introduction.**

46 Selecting a mRNA for translation is a well-studied process requiring several consecutive
47 steps and the contribution of more than 12 initiation factors. In virtually of eukaryotes,
48 the majority of mRNAs initiate translation via a canonical cap-dependent mechanism,
49 when the initiation factor eiF4E recognises a cap structure at the 5’ end of the mRNA.
50 Then, a cap-binding complex formed by eiF4E, eiF4G and eiF4A allow further
51 recruitment of a ternary complex (eIF2/GTP/$tRNA^{met}$), the small ribosome subunit, and
52 the additional initiation factors eIF3, eIF1 and eIF1A. This big complex then scans the
53 mRNA until an ATG codon is found, after which the large ribosomal subunit is bound,
54 and the elongation phase starts (Muñoz and Castellano, 2012).
55 Although most of the plant translation machinery resembles that of other eukaryotes,
56 differences found in recent years in some of the components of this important process
57 suggest that plants regulate their translation in unique ways. (Browning, 2004). Plants,
58 for example, present a second eIF4F factor, called eIF(iso)4F, and certain features in the
59 mRNAs allow different transcripts to interact with one or the other factor (Mayberry et
60 al, 2009). In Arabidopsis, the double mutant of the two existing eIF(iso)4F isoforms show
61 strong developmental phenotypes, (Lellis et al, 2010). General inhibition of translation is
62 absent in this mutant, indicating that these factors are probably required for appropriate
63 expression of specific genes that may participate in the regulation of plant growth and
64 development.
65 A conserved mechanism for regulation of translation is the one dependent on the
66 phosphorylation of the eIF2α factor by specific kinases. This phosphorylation step
67 prevents the interaction of eIF2-GDP with the eIF2B factor to regenerate GTP, thus
68 blocking protein synthesis. Simultaneously, at least in yeast and mammals, a key
69 transcription factor (GCN4 in yeast, ATF4 in mammals) is translationally derepressed
70 when eIF2α is phosphorylated, to enhance expression of stress recovery genes.
71 (Hinnebusch, 2005) This mode of regulation has been generally associated with cellular
72 stresses, where general inhibition of the energy-consuming translation is needed in order
73 to conserve resources and to initiate a reconfiguration of gene expression to effectively
74 manage stress conditions (Castilho et al, 2014). Until recently, all the evidence indicated
75 that this important mechanism of translational regulation could be also operating in
76 plants. A functional eIF2α kinase (GCN2 for *General Control Non-repressive 2*) does
77 exist in virtually all plant genomes analysed, presenting all the structural domains of

78 mammals or yeast eIF2α-kinases needed to perform its function. In fact, the Arabidopsis
79 gene (*AtGCN2*) complements the yeast *gcn2* mutant (Zhang et al. 2003), and an
80 Arabidopsis *gcn2* knock-out mutant line is unable to phosphorylate eIF2α (Zhang et al.
81 2008). Moreover, like mammals and yeast eIF2α-kinases, the Arabidopsis GCN2 protein
82 interacts with uncharged tRNAs and has activity on different eIF2α isoforms (Li et al.
83 2013). However, although *At*GCN2 phosphorylates eIF2α under many different stresses
84 (Lageix et al, 2008), it has recently been shown that eIF2α phosphorylation does not
85 correlate with global protein synthesis inhibition (Izquierdo et al 2018). This fact, together
86 with the absence of GCN4 homologs, suggest that regulation of translation via eIF2α may
87 be a minor pathway in plants.
88 The role of the two main GCN2-interacting proteins (GCN1 and GCN20) is also elusive
89 in plants. In yeast and mammals, activation of GCN2 requires binding to GCN1, forming
90 a complex with the ATP-binding cassette protein GCN20, both attached to ribosomes. As
91 a result, yeast *gcn1* and *gcn20* knock-out strains are deficient in GCN2 activation and
92 eIF2α phosphorylation (Marton et al. 1993). In Arabidopsis, a GCN1 homolog (also
93 called ILITHYIA or NOXY7) (Monaghan et al, 2010; Izquierdo et al, 2018) interacts
94 with GCN2, and an Arabidopsis *gcn1* mutant is unable to phosphorylate eIF2α (Wang et
95 al, 2016; Faus et al, 2018). However, *gcn2* and *gcn1* mutants present very different
96 phenotypes (Faus et al, 2018). Whereas *gcn2* mutants are indistinguishable from wilt
97 type, *gcn1* alleles present diverse developmental and stress-related defects, specially the
98 stronger ones, (Monaghan et al, 2010; Faus et al, 2018), evidencing a GCN2-independent
99 function for GCN1 in Arabidopsis, probably related also to the control of protein
100 synthesis, as recently demonstrated by Izquierdo et al, 2018.
101 In yeast, GCN20 is another positive effector of GCN2, facilitating the activation of the
102 kinase by uncharged tRNA on translating ribosomes. The N-terminal domain of GCN20
103 binds to the central eIF3-like domain of GCN1, thus modulating its activity (Marton et
104 al, 1997; Garcia-Barrio et al, 2000). GCN20 belongs to a subfamily of ATP-binding
105 cassettes (ABC) without transmembrane domains, whose members are conserved among
106 eukaryotes. There are 26 of those genes in Arabidopsis, five of them falling into a clearly
107 differentiated cluster (ABC-F) whose closest homolog in yeast is the GCN20 protein
108 (Sanchéz-Fernández et al, 2001). One of those genes, ABCF3, named SCORD5 (for
109 "*Susceptible to Coronatine (COR)-Deficient*"), was initially identified in a screen looking
110 for Arabidopsis mutants that could rescue the virulence of COR-deficient mutant bacteria
111 (Zeng et al, 2011). SCORD5/GCN20 has been recently shown to interact with GCN1 in

Arabidopsis; however, it is not essential for eIF2α phosphorylation (Izquierdo et al, 2018), and a common role with GCN1 in its GCN2-independent function has been suggested. *scord5* (henceforth *abcf3*/*gcn20*) knock-out mutant present similar phenotypes to *gcn1* mutants. For example, both *abcf3/gcn20* and *gcn1*, but not *gcn2*, show seedling yellowness and are unable to close stomata after bacterial infection (Zeng et al, 2011) and were sensitive to boric acid and antimycin A (Izquierdo et al, 2018). The three mutants, however, are susceptible to the amino acid synthesis inhibitor CHL, but only *gcn2* and *abcf3/gcn20* are sensitive to paromomycin. The participation of the three GCN genes in the same process of regulation of translation is still under debate. Recently, we showed that GCN1 was involved in chloroplast biogenesis and root development, independent of GCN2 (Faus et al, 2018). In this work we describe phenotypic and molecular assays that reinforce the common function of the GCN20 protein (ABCF3) with GCN1 in biological processes where GCN2 seems not to be involved.

MATERIALS AND METHODS

Plant growth

The following genotypes were used in this study: wild-type Col-0, Col-7, *gcn1* (*ila3* (SALK_ 041123), *gcn2.2* (SALK_032196), *abcf1* (SAIL 412-A12), *abcf2* (SALK_018778C), *abcf3* (*scord5-1* from Dr. Shang Yang He lab, Michigan State University, *abcf4* (SALK_202649C) and *abcf5* (SALK_113472C).

Seeds were pretreated in 70% ethanol for 20 min, surface-sterilized in 2.5% bleach for 5 min and washed with distillated water at least three times. After stratification at 4°C in the dark for 3 days, seeds were sown on 0,9% agar-containing 0,4% MS Salts, 1% sucrose, pH 5.7, and grown at 23°C with a 16-h-light/8-h-dark cycle.

For phenotyping at seedling stage, MS medium was supplemented with abscisic acid (0,8 µM), paraquat (0,7 µM), NaCl (125 mM) or acetic acid (4 mM) and percentage of seedlings with green cotyledons was calculated after 7-9 days.

For experiments in adult plants, 7-day-old plantlets were transferred to soil and grown on a soil mix of 25% perlite, 25% vermiculite and 50% peat moss, in environmental growth chambers under long-day (16 hours light at 21°C and 8 hours dark at 19°C) photoperiod cycle, with a light intensity of 150 µmol m−2 s−1. Visual inspection was followed during the next four weeks.

P-eIF2α western blots

145 For these experiments, 10-days-old seedlings grown on MS media were used. Seedlings
146 were UV-C exposed for around 25 minutes (9000 energy x2 + 4500. Stratalinker 1800)
147 and collected immediately. Protein was extracted using the P-EIF2α extraction buffer
148 described elsewhere (Zhang et al. 2008). Proteins (20 µg) were loaded in a 10% SDS-
149 PAGE gel and inmunoblotting was performed using Phospho-eIF2α (Ser51) antibody
150 (Cell Signalling) at a 1:2000 dilution and a secondary ECL anti-rabbit IgG horseradich
151 peroxidase-linked whole antibody (GE Healthcare) at a 1:10.000 dilution and visualized
152 using a chemiluminescence system.

153 **Transmission electron microscopy (TEM)**

154 Arabidopsis plants were grown on the greenhouse under long-day conditions for 30 days.
155 For TEM, LR-white resin inclusion was performed fixing Arabidopsis leaves with
156 glutaraldehyde 2.5%, washed three times (5 min each) with phosphate buffer 0.1M
157 pH=7.2, and post-fixed with Osmium for 2h. After three washes with water (5 min each),
158 they were sequentially dehydrated in EtOH 30%-90% and incubated for 2h in LR-white
159 resin in EtOH 90%, LR-white resin in EtOH 100% and 100% LR-white resin. Ultrathin
160 slides (60nm) were stained with 2% uranil acetate and plumb prior to viewing by
161 transmission EM (TEM) using a Jeol JEM1010 microscope at 60kV. Images were
162 acquired with a digital camera AMT RX80 (8Mpx).

163

164 **Microarray experiments**

165 Total RNA was extracted from 20-days-old Col7 and *abcf3/gcn20* plants. Transcriptome
166 analysis was done using the Agilent Arabidopsis (V4) Gene Expression Agilent 4x44
167 Microarray. Three biological replicates of *abcf3/gcn20* versus Col-7 wild-type
168 comparisons were performed. RNA integrity was assessed using the 2100 Bioanalyzer
169 (Agilent). 0.5 µg RNA was amplified and labeled with the Agilent Low Input Quick Amp
170 Labeling Kit. An Agilent Spike-In Kit was used to assess the labeling and hybridization
171 efficiencies. Hybridization and slide washing were performed with the Gene Expression
172 Hybridization Kit and Gene Expression Wash Buffers, respectively. After washing and
173 drying, slides were scanned in a GenePix 4000B microarray scanner, at 5 µm resolution
174 and using the double scanning. Image files were analyzed with the Feature Extraction
175 software 9.5.1. Interarray analyses were performed with GeneSpring 11.5 software. To
176 ensure a high-quality data set, control features were removed, and only those for which
177 the 'IsWellAboveBG' parameter was 1 in at least two out of three replicates were

178 selected. To identify significantly expressed genes, a one-class significant analysis of
179 microarrays (SAM) test (Tusher et al. 2001) was performed with adjustment according to
180 Benjamini and Hochberg's method. Features were selected only if q value was below 1
181 after correction for multiple testing and expression ratio was greater than twofold
182 different, for those genes having a valid value in the three replicates. Gene Enrichment
183 analysis on Gene Ontology tools was performed using agrogo v2.2 (Tian et al, 2017), and
184 a representative subset of the enriched GO-terms was obtained using a simple clustering
185 algorithm (ReviGO) that relies on semantic similarity measures (Supek et al. 2011).
186 These microarrays data have been included in the GEO Omnibus database with the
187 reference numbers GSE136779
188

189 **RESULTS**

190 **None of the five soluble Arabidopsis ABC transporters of the GCN subfamily is**
191 **essential for eIF2α phosphorylation.**

192 From the 26 ORFs of Arabidopsis encoding ABC proteins lacking contiguous
193 transmembrane spans (Sanchéz-Fernández et al, 2001), the five members of the ABCF
194 subfamily fall into a clade based on sequence homology, with two nucleotide binding
195 domains but without transmembrane spans. The five proteins share homology with the
196 yeast GCN20 protein and other related proteins on the two ABC transporter domains, but
197 present different N-terminal domains. ABCF3 (putative GCN20) binds GCN1 in
198 Arabidopsis but is not necessary for eIF2α phosphorylation (Izquierdo et al, 2018). In
199 order to discard the possibility that any of the other GCN20-like proteins of the GCN-like
200 ABC-transporters clade could be involved in GCN2 activation, T-DNA knock-out mutant
201 lines of the five gcn20-like genes (*abcf1,abcf2,abcf3,abcf4 and abcf5)* were assayed for
202 phosphorylation of the GCN2 substrate: eIf2α. Seedlings were treated with UV-C light,
203 known to phosphorylate eIf2α in Arabidopsis in a GCN2-dependent manner (Faus et al,
204 2018). Exposing Arabidopsis seedlings to UV-C results in a clear activation of GCN2 as
205 detected by eIf2α phosphorylation (Fig.1). As reported by Izquierdo et al, 2018
206 phosphorylation is also observed in the *abcf3* mutant. Our results show that the other four
207 mutant lines (*abcf1,2,4 and 5)* were also able to phosphorylate eIF2α, suggesting that
208 none of the five genes of the Arabidopsis GCN20 clade is essential for GCN2 activation.

209 **Plant response under abiotic stresses is shared by *abcf3* and *gcn1*.** To evaluate the
210 consequences of the loss of function of the different GCN genes in the response to plant
211 abiotic stress, we compared the phenotype of wild type plants with that of knock-out lines
212 of gcn1(*ila3*), gcn2(*gcn2.2*), *abcf3* and the other four gcn20-like genes under different
213 abiotic conditions. As shown in Fig. 2, both *gcn1* and *abcf3* seedlings were clearly
214 resistant to paraquat as compared to wild type, but no *gcn2* nor any of the other gcn20-
215 like mutants (except *abcf5*). In contrast, only *gcn1* and *abcf3* were more sensitive than
216 wild type to treatment with abscisic acid, whereas *gcn2* and the other four gcn20-like
217 mutants respond similarly to wild type. Similarly, in response to NaCl, *gcn1* and *abcf3*,
218 but no *gcn2* were more sensitive than wild type. In this case, the other four gcn20-like
219 mutants respond differently, being *abcf1, abcf2* and *abcf5* more sensitive than wild type,
220 and *abcf4* responding similar to wild type. Only in medium supplemented with acetic
221 acid, however, the response of *abcf3* and *gcn1* differs, being the first sensitive and the
222 second resistant to the stress. In summary, these data suggest a coordinated response of
223 GCN1 and ABCF3 to most of these abiotic stresses, which seems independent of GNC2.
224 The phenotype of the other four gcn20-like mutants is not correlated with *gcn1* in most
225 of the stresses assayed, and the functional link between those genes deserve further
226 studies.

227

228 **ABCF3 and GCN1 are essential for chloroplast biogenesis.**

229 Contrary to *gcn2* plants, which are indistinguishable from wild type, *gcn1* plants are
230 yellow to light green in colour, especially in emerging leaves (Monaghan et al, 2010).
231 *abcf3* and the other gcn20-like mutants were grown in the greenhouse and their
232 phenotypes were compared to *gcn1* and *gcn2*. As observed in Fig. 3a, only *abcf3*, but not
233 the other four knock-out gcn20-like mutants presents yellow leaves, reinforcing a link
234 between *gcn1* and *abcf3/gcn20*, and dismissing a role of any of the other gcn20-like genes
235 in these developmental phenotypes observed in the *gcn1* mutant. We have previously
236 reported that *gcn1* mutants, but not *gcn2,* present altered root and chloroplast
237 development (Faus et al, 2018). Recently, it was described that ABCF3, similarly to
238 GCN1, is also involved in root development, by modulating DNA damage repair (Han et
239 al, 2018). To know if the chloroplast defects of *gcn1* were also shared by *abcf3,* electron
240 microscopy experiments were performed. As shown in Fig. 3b, wild-type leaves

241 contained fully developed chloroplasts with internal thylakoid membranes stacked into
242 grana layers. In contrast, and similarly to *gcn1* chloroplasts (Fig. 3c), *abcf3* chloroplast
243 contained a poorly developed thylakoid network with wide luminal areas between the
244 thylakoid membranes (Fig. 3d). We have previously reported that *gcn2* chloroplasts
245 present a similar appearance to wild type, with a well-established thylakoid structure and
246 correctly stacked grana system (Faus et al, 2018). These results suggest that the ABCF3
247 protein is necessary for the correct development of the thylakoid network in the
248 chloroplasts, a role that could be performed together with GCN1 but independent of
249 GCN2.

250 ***abcf3* and *gcn1* mutant plants share transcriptomic profiles.**

251 To examine to what extend the consequences of GCN20 loss-of-function are shared by
252 the loss-of–function of GCN1, microarray experiments comparing gene expression of
253 two-weeks old wild type and *gcn20/abcf3* seedlings were performed, and differentially
254 expressed genes were contrasted to those reported by Faus et al, 2018. Seedlings of wild
255 type and *gcn2* of the same age do not differ in their transcriptomic profiles (Faus et al,
256 2015). Following the same criteria as in Faus et al, 391 genes were considered upregulated
257 and 215 downregulated in the *gcn20/abcf3* mutant (Supplemental table 1). Defense
258 response and photosynthesis-related functional categories were enriched upon
259 downregulated genes in *gcn1* (Faus et al, 2018). Enrichment analysis upon the genes
260 downregulated in *gcn20/abcf3* also identified categories related to defense, such as
261 defense response (FDR $1.64e^{-16}$), or more specifically, incompatible interaction (FDR
262 $7.1e^{-06}$) or response to salicylic acid (SA) (FDR $6.26e^{-08}$), and photosynthesis, light
263 reaction (FDR, 0.03), indicating that loss-of-function of any of the two genes
264 compromises similar biological processes (Supplemental table 2, Fig. 4a). Indeed, as
265 shown in Fig. 4b, more than 50% of the genes downregulated in *gcn20/abcf3* were also
266 downregulated in *gcn1(ila3).* Among the genes downregulated in *gcn20/abcf3*, we found
267 several cysteine-rich receptor-like protein kinases (CRKs), playing relevant roles in the
268 regulation of pathogen defense and programmed cell death, ALD1, involved in pipecolin
269 acid production, relevant for systemic acquired resistance (SAR) signaling, the SAR-
270 marker PR1, the PTI marker FRK1 or several WRKY transcription factors involved in
271 defense responses. Similarly, photosystem II-related genes such as LHB1B1, Lhcb2.4, or
272 the chlorophyll binding protein D1, a part of the reaction center PSBA, as well as the FED
273 A major leaf ferredoxine, were also downregulated in *gcn20/abcf3.* (Supplemental table

274 1). The list of *gcn20/abcf3* upregulated genes was enriched in categories such as response
275 to chitin (FDR, $1.6e^{-14}$), response to heat (FDR, 0.9 e^{-4}), and response to oxidative stress
276 (FDR, 0.0014), (Supplemental table 2, Fig. 4a) all coincident with those observed in *gcn1*
277 analysis. Accordingly, 33% of the genes were overexpressed in both mutants. Other
278 categories were enriched only among the *gcn20/abcf3* overexpressed genes, including
279 regulation of transcription (FDR, $1.6e^{-09}$), or response of jasmonic acid (FDR, 0.1 e^{-4})
280 among others. All these results clearly indicate that *gcn20/abcf3* loss-of-function alters
281 the transcriptomic profile of the plant in many processes shared by *gcn1*.

282 In Arabidopsis, GCN20 and GCN1 are required for bacterium-triggered stomata closure.
283 The *gcn20/abcf3* mutation affected MAMP-induced stomatal closure, but not SA- or
284 ABA-induced stomatal closure, suggesting that GCN20 likely acts early in the stomatal
285 closure response pathway (Zheng et al, 2011). Among the genes that are down-regulated
286 in both mutants, we found ACD6 (accelerated cell death 6). This gene was nearly four
287 times repressed in *gcn20/abcf3* and *gcn1*. ACD6 positively controls the membrane levels
288 of the flagellin receptor FLS2 (Tateda et al. 2014, Zhang et al. 2014), essential for
289 stomatal response (Zheng et al, 2010). In addition, the cysteine-rich receptor-like protein
290 kinase CRK4 was found 5.5 times less expressed in *gcn20/abcf3*, and it was found 3.2
291 times less expressed in *gcn1*. CRK4 interacts with FLS2, and it has been described that
292 CRK4 overexpression lines present enhanced stomatal immunity (Yeh et al, 2016). These
293 results could indicate that the role of ABCF3 and GCN1 in stomata closure response could
294 be mediated by FLS2 levels.

295 **DISCUSSION**

296 Initially identified in yeast as a suppressor mutation that overcomes the toxic effect of a
297 constitutive GCN2 allele, the GCN20 gene is a positive effector of the eIF-2a kinase
298 activity of GCN2, and it forms, together with GCN1, a protein complex required for the
299 activation of GCN2 by uncharged tRNA on translating ribosomes (Marton et el, 1997).
300 The whole system is an important hub controlling mRNA translation and stress adaptation
301 in yeast, *C. elegans* or mammals (Hirose and Horvitz, 2014), and the conservation of
302 these three proteins initially suggested a conserved regulatory mechanism in plants.
303 However, recent results obtained in Arabidopsis indicate a minor relevance of GCN2 and
304 P-eIF2α in the overall inhibition of translation (Izquierdo et al, 2018): in essence, eIF2α
305 is phosphorylated by GCN2 in many stress conditions, but the exact role of this

306 phosphorylation is still unknown. Mechanistically, GCN1 is required for eIF2α-
307 phosphorylation in Arabidopsis, but unexpectedly, ABCF3, a protein homologous to the
308 yeast GCN20, is not (Izquierdo et al, 2018). We also dismissed that loss-of-function of
309 the other GCN20-like isoforms could compromise eIF2α-phosphorylation. The five
310 members of the GCN20 subfamily in Arabidopsis contain two ABC domains without a
311 transmembrane domain but differ markedly in the N-termini. Non-membrane ABC
312 proteins are known to be involved in translation. The human ABC50 protein, for instance
313 (the only member of the ABCF family, apart from GCN20, which has been characterized
314 in detail), binds to ribosomes and interacts with the eukaryotic initiation factor eIF2,
315 which plays a key role in translational initiation control. Moreover, since it is only the N-
316 terminal of GCN20 that is required to support the function of GCN2 in yeast (Marton et
317 al, 1997), and the five Arabidopsis ABCF proteins differ in their N-termini, it seems
318 unlikely that this phenotype is explained by gene redundancy. Either GCN1 can perform
319 GCN2 activation without the help of GCN20, or another protein is performing this role
320 in Arabidopsis. On the other hand, GCN1 interacts with ABCF3, and they coordinately
321 regulate stress responses through translational regulation, but independent of GCN2
322 (Izquierdo et al, 2018). The hypothesis that GCN1 could have a GCN2-independent
323 function is reinforced by the different phenotype of *gcn2* and *gcn1* loss-of-functions
324 alleles. Whereas *gcn2* plants are indistinguishable from wild type, *gcn1* alleles present
325 clear stress-related and developmental phenotypes (Monaghan et al, 2010; Faus et al,
326 2018). Zeng et al, 2011 reported that *gcn1* and *gcn20(scord5)* alleles are unable to close
327 stomata after bacterial infection, and are susceptible to pathogen attack. Later, Izquierdo
328 et al, 2018 wider the similarities between these two proteins by examining their response
329 after boric acid treatment, and suggested a coordinated role of these two proteins in
330 protein synthesis. Here, the similar response after paraquat, NaCl and abscisic acid
331 treatments and the similar defects in chloroplast development further reinforces a
332 coordinated action of GCN1 and GCN20(ABCF3) in response to environmental stresses,
333 independent of the action of GCN2. Tolerance to paraquat, a potent superoxide producer,
334 could be explained as the category "response to oxidative stress" was highlighted upon
335 the gene upregulated in *gcn20/abcf3,* as it happened with *gcn1* (Faus et al, 2015). In
336 particular, the chloroplastic copper/zinc superoxide dismutase SOD1 was three-fold
337 induced in *gcn1*, and SOD2 was two-fold induced in *gcn20/abcf3*. However, since
338 chloroplast is impaired in the two mutants, electron transport should be weakened as well.
339 Thus, the capacity of the mutants to generate ROS from paraquat should have been

340 reduced. It is hard to conclude what is the cause of tolerance without further evidence.
341 The fact that loss of function of any of these two proteins confers tolerance to paraquat
342 but susceptibility to NaCl is intriguing, as oxidative stress is an important component
343 involved in salinity-induced damage (Moradi and Ismail 2007), and the accumulation of
344 NaCl is followed by an increase of superoxide and H_2O_2 (Mishra et al, 2013). Considering
345 that both mutants are sensitive to ABA, that mimics osmotic stress, we suggest that the
346 osmotic component of NaCl toxicity could be the cause of the observed phenotypes.

347 Transcriptomes of *abcf3*/*gcn20* and *gcn1* support other previously reported phenotypic
348 results. Defects in chloroplast biogenesis (Faus at el, 2018) is evidenced by the down-
349 regulation of genes related to photosynthesis and the similar defects observed in *abcf3*
350 and *gcn1* chloroplasts. The increased expression in *gcn1* tissues of genes involved in
351 removal of superoxide could indicate a context of oxidative stress in these mutants.
352 Mutants in the ferredoxin-NADP(+)-oxidoreductase gene, that also display a highly
353 chloroplast-deficient phenotype also induce ROS-scavenging systems to protect damaged
354 chloroplasts. (Lintala et al., 2012). A similar mechanism could also be occurring in *gcn1*
355 and *abcf3* leaves as a common response to protect defective tissues that result from the
356 mutation.

357 Also, defects in immunity (Monaghan et al, 2010) relates with the shared down-regulation
358 of genes involved in defense responses, mainly in salicylic-related responses. Strikingly,
359 these include the ACD6 and the CRK4 genes, two genes whose down-regulation is known
360 to directly affect the function of the FLS2 receptors. *gcn1* and *abcf3* mutants share their
361 impossibility to close stomata after bacterial infection (Zheng et al, 2011). This response
362 is triggered by the well-characterized pathogen-associated molecular pattern (PAMP)
363 flg22 (a peptide derived from bacterial flagellin). Flagellin (o flg22) is recognized in the
364 plant cell by the flagellin-receptor FLS2, which activates the signaling cascade involved
365 in pathogen- or PAMP-induced stomatal closure (Zeng et al, 2010). A major function for
366 ACD6 is to regulate the plasma membrane pool of FLS2 (Zhang et al, 2014). ACD6 and
367 FLS2 form a protein complex in the plasma membrane, and in benzothiadiazole-treated
368 plants that lacked ACD6 (*acd6-2* mutants), FLS2 failed to show increased levels in this
369 compartment. A role for ACD6 in FLS2-mediated signalling is also suggested by the
370 reduced transcriptional response of the *acd6-2* loss-of-function mutant to the FLS2 ligand
371 flg22. FLS2 pools and downstream responses could be also diminished in *abcf3* or *gcn1*
372 plants, in which ACD6 is downregulated. In addition, it has been reported that

overexpression of cysteine-rich receptor-like kinase 4 (CRK4) present a defective Pst DC3000-mediated stomatal reopening (Yeh et al, 2015), indicating a role of this gene early during the activation of this PTI response. Co-IP assays indicated that CRK4 associates with FLS2, although in this case the mechanisms are unknown. The down-regulation of CRK4 levels in *abcf3* and *gcn1* mutants could again affect the proper function of the FLS2-mediated stomatal closure after infection.

The response to jasmonic acid was enriched in the *abcf3* overexpressed genes, and this could also explain the repression of the salicylic responses observed in this mutant. The antagonistic effect of jasmonic acid (JA) and salicylic pathways is well documented. Some pathogens disable SA signalling, as the bacterial toxin coronatine (COR) mimics JA. COR can activate the JA signaling pathway, and enhance bacterial virulence by suppressing SA-mediated defence through hormonal crosstalk. (Kazan and Lyons, 2014). The higher expression in *abcf3* mutants of the MYC2 transcription factor, that activates the jasmonic acid pathway and is involved in the salicylic acid crosstalk (Zheng et al, 2012; Du et al, 2017)), could be responsible of the inhibition of the salicylic pathway and the lower expression of defence genes in *abcf3*.

In summary, phenotypic and molecular data further confirm the functional association of GCN1 and GCN20 in a GCN2-independent manner and reinforces the idea of a new function for these two proteins. The assays of *gcn1* and *abcf3* mutants after Pst DC3000 infection (Izquierdo et al, 2018) supports a role for GCN1 and GCN20(ABCF3) in the preinvasive response to bacterial infection, in which these proteins could regulate translation of specific proteins. However, the basal transcriptomic profiles of both mutants indicate that this level of regulation could also be determinant for this phenotype.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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508

509 **Fig 1** Arabidopsis GCN20-like proteins are not essential for eIF2α phosphorylation.
510 Western blot assaying eIF2α phosphorylation on wild-type (Col-0 or Col-7), *abcf1,2,3,4*
511 and *5* mutants and *gcn1* (*ila-3*) mutant seedlings, mock-treated and treated with UV-C to
512 induce phosphorylation. A differential band corresponding to P-eIF2α is shown by
513 arrows. Equal amount of protein (20µg) was loaded in a 10% SDS-PAGE gel. LC:
514 Loading control.

515 **Fig 2** *abcf3/gcn20* and *gcn1* mutants share similar phenotypes under abiotic stress
516 conditions. Graphics show the percentage of seedlings with green cotyledons after
517 growing 7 days on MS medium supplemented with 0,7 µM paraquat (top left), 125 mM
518 NaCl (top right) or 4 mM acetic acid (bottom left) and after growing 9 days on MS
519 medium supplemented with 0,8 µM ABA (bottom right). Bars represent mean and
520 standard error of three biological replicates. * Indicate statistical differences after t-test
521 (p-value= 0,05) between the mutant and its corresponding wild type.

522 **Fig 3** *abcf3/gcn20* mutant has defective chloroplast development. a) Representative
523 image of the rossette leaves of wild type (Col-0 or Col-7), *abcf1-5*, *gcn1* (*ila-3*) and *gcn2*
524 (*gcn2.2*) plants after growing 4 weeks in the greenhouse. Only leaves of *gcn1 and*
525 *abcf3/gcn20* mutants are pale green. b) Transmission electron microscopy images of Col-
526 0, *gcn1* and *abcf3*/*gcn20* chloroplasts showing very few thylakoids in the mutant
527 chloroplasts. Plants were grown for 30 days in the greenhouse under long-day conditions.
528 Scale bars: 800 nm (a,b) or 500 nm (c). T: Thylakoids.

529 **Fig 4** *gcn20* and *gcn1* mutant plants share transcriptomic profiles. a) Representative Gene
530 Ontology (GO) categories enriched in *abcf3/gcn20* overexpressed and underexpressed
531 genes. The scatterplot shows the cluster representatives (terms remaining after the
532 redundancy reduction) in a two-dimensional space derived by applying multidimensional
533 scaling to a matrix of the GO terms' semantic similarities, according to REVIGO
534 software. Colour scale (log10 p-value). Bubble colour indicates uniqueness of a particular
535 GO term (legend in upper right-hand corner), uniqueness is calculated in REVIGO as the
536 negative of average similarity of a term to all other terms.; bubble size indicates the
537 frequency of the GO term in the GO database (bubbles of more general terms are larger).
538 (Supek et al. 2011). b) Venn diagrams showing number of genes overexpressed and
539 underexpressed in *gcn1(ila3)* (Faus et al, 2018) and *abcf3*/*gcn20.*

540 **Supplemental Table 1** Differentially-expressed genes in the transcriptomic experiment
541 comparing wild-type and *gcn20* seedlings. Columns correspond to: B. numerator after
542 SAM test (average fold-change) C. q-value D-F. Independent ratios gcn20/wild-type in
543 the three replicates

544 **Supplemental Table 2** Gene ontology categories considered enriched among the genes
545 differentially expressed in the transcriptomic experiment comparing wild-type and *gcn20*
546 seedlings.