

1 **The *Arabidopsis* ERF transcription factor ORA59 coordinates jasmonic**
2 **acid- and ethylene-responsive gene expression to regulate plant immunity**

3
4 **Young Nam Yang¹, Youngsung Kim¹, Hyeri Kim¹, Su Jin Kim¹, Kwang-Moon Cho², Yerin**
5 **Kim³, Dong Sook Lee^{1,6}, Myoung-Hoon Lee^{1,7}, Soo Young Kim⁴, Jong Chan Hong⁵, Sun**
6 **Jae Kwon², Jungmin Choi³, and Ohkmae K. Park^{1,*}**

7
8 ¹Department of Life Sciences, Korea University, Seoul 02841, Korea

9 ²Molecular Diagnosis Division, AccuGene, Incheon 22006, Korea

10 ³Department of Biomedical Sciences, Korea University College of Medicine, Seoul 02841,
11 Korea

12 ⁴Department of Biotechnology and Kumho Life Science Laboratory, College of Agriculture
13 and Life Sciences, Chonnam National University, Gwangju 61186, Korea

14 ⁵Division of Life Science, Plant Molecular Biology and Biotechnology Research Center,
15 Gyeongsang National University, Jinju 52828, Korea

16 ⁶Present address: Animal and Plant Quarantine Agency, Foot and Mouth Disease Research
17 Division, Gimcheon 39660, Korea

18 ⁷Present address: Genuine Research, Seoul 06040, Korea

19 *Correspondence: omkim@korea.ac.kr

20
21
22 Corresponding Author:

23 Ohkmae K. Park

24 Department of Life Sciences

25 Korea University

26 Anam-dong, Seongbuk-gu

27 Seoul 02841, Korea

28 Phone: +82-2-3290-3458

29 E-mail: omkim@korea.ac.kr

30 **Abstract**

31

32 Jasmonic acid (JA) and ethylene (ET) signaling modulate plant defense against necrotrophic
33 pathogens. These hormone pathways lead to transcriptional reprogramming, which is a major
34 part of plant immunity and requires the roles of transcription factors. ET response factors are
35 responsible for the transcriptional regulation of JA/ET-responsive defense genes, among which
36 ORA59 functions as a key regulator of this process and has been implicated in the JA-ET
37 crosstalk. Here, we identified the ERELEE4 as an ORA59-binding *cis*-element, in addition to
38 the well-characterized GCC box, demonstrating that ORA59 regulates JA/ET-responsive genes
39 through direct binding to these elements in the gene promoters. Notably, ORA59 exhibited
40 differential preference for the GCC box and ERELEE4, depending on whether ORA59
41 activation is achieved by JA and ET, respectively. Our results provide insights into how ORA59
42 can generate specific patterns of gene expression dynamics through JA and ET hormone
43 pathways.

44

45

46 **Keywords:** *Arabidopsis*, ORA59, transcription factor, ERELEE4, GCC box, ethylene,
47 jasmonic acid, plant immunity

48 **Introduction**

49

50 In nature, plants encounter a wide range of microbial pathogens with varying lifestyles and
51 infection strategies. Upon pathogen recognition, plants rapidly activate defense responses, and
52 the levels of resistance are influenced by hormone actions (De Vos et al., 2005; Pieterse et al.,
53 2009). Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are primary defense
54 hormones that trigger immune signaling mechanisms (Dong, 1998; Pieterse et al., 2012).
55 Classically, SA signaling enhances resistance against biotrophic and hemibiotrophic pathogens
56 such as *Hyaloperonospora arabidopsidis* and *Pseudomonas syringae*, whereas JA and ET
57 signaling activate resistance against necrotrophic pathogens such as *Alternaria brassicicola*
58 and *Botrytis cinerea* (Feys and Parker, 2000; Glazebrook, 2005). Antagonism between SA and
59 JA/ET and synergism between JA and ET have been mostly observed in studies of plant
60 immunity, although there is evidence of positive interactions between them (Kim et al., 2013;
61 Koornneef and Pieterse, 2008; Penninckx et al., 1998; Thomma et al., 1998). These hormone
62 signaling pathways are interconnected in a complex network and their crosstalk enables plants
63 to tailor defense responses efficiently (Bostock, 2005; Kunkel and Brooks, 2002; Spoel and
64 Dong, 2008).

65 JA and ET modulate diverse developmental processes and defense responses in plants
66 (Broekgaarden et al., 2015; Joo and Kim, 2007; Zhu and Lee, 2015). Their signaling pathways
67 work by de-repression mechanisms. MYC2/3/4 transcription factors play essential roles in JA
68 signaling, and in the absence of JA, remain in repressed states by binding to transcriptional
69 repressors jasmonate ZIM-domain (JAZ) proteins (Chini et al., 2007; Thines et al., 2007). JA
70 promotes the interaction between JAZs and the F-box protein coronatine insensitive 1 (COI1),
71 resulting in degradation of JAZs and de-repression of MYCs (Katsir et al., 2008; Sheard et al.,
72 2010; Yan et al., 2009). The activated MYCs then regulate gene expression and various JA

73 responses (Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011). ET insensitive 2
74 (EIN2) and EIN3 are key positive regulators of ET signaling (Alonso et al., 1999; Chao et al.,
75 1997). In the absence of ET, ET receptors activate the Raf-like serine/threonine kinase
76 constitutive triple response 1 (CTR1), which phosphorylates EIN2 to repress its activity
77 (Kieber et al., 1993). EIN3 and its homolog EIN3-like 1 (EIL1) are also targeted for
78 degradation by EIN3-binding F-box protein 1 (EBF1) and EBF2 (Guo and Ecker, 2003;
79 Potuschak et al., 2003). ET binding to ET receptors deactivates CTR1, which is followed by
80 de-repression of EIN2 (Chao et al., 1997). In the activation process, EIN2 is cleaved and its C-
81 terminal fragment translocates into the nucleus and inhibits EBF1/2, promoting EIN3/EIL1
82 accumulation (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). EIN3/EIL1 further activate
83 downstream genes, including the ET response factor (ERF) family transcription factors (Chang
84 et al., 2013; Solano et al., 1998). EIN3/EIL1 and ERFs regulate ET-mediated gene expression.

85 Plant defense against necrotrophic pathogens requires JA and ET, and synergistic and
86 interdependent interactions between JA and ET have been described (Koornneef and Pieterse,
87 2008; Thomma et al., 1998). ERFs are important regulators of the JA-ET crosstalk, and in
88 particular, ERF1 and octadecanoid-responsive arabidopsis 59 (ORA59), belonging to the group
89 IX ERF family, have been suggested to act as integrators of ET and JA signaling (Lorenzo et
90 al., 2003; Pré et al., 2008b). The expression of *pathogenesis-related (PR)* genes such as *plant*
91 *defensin 1.2 (PDF1.2)* and *basic chitinase (b-CHI)* was synergistically induced by JA and ET,
92 and abolished in JA-insensitive *coil* and ET-insensitive *ein2* mutants, while depending on
93 ERF1 and ORA59 (Lorenzo et al., 2003; Penninckx et al., 1998; Pré et al., 2008b). Analysis of
94 the *PDF1.2* promoter indicated that ERF1 and ORA59 induce *PDF1.2* expression through
95 direct binding to GCC boxes in the *PDF1.2* promoter (Zarei et al., 2011). Like other *PR* genes,
96 the expression of *ERF1* and *OAR59* themselves exhibited a synergistic response to JA and ET,
97 which was impaired in *coil* and *ein2* mutants. The role of ERF1 and ORA59 in defense has

98 been revealed in *ERF1*- and *ORA59*-overexpressing plants displaying enhanced resistance to
99 necrotrophic pathogens (Berrocal-Lobo et al., 2002; Pré et al., 2008b; Kim et al., 2018).

100 *ERF1* and *ORA59* have been determined to be regulated by EIN3 and their JA- and ET-
101 responsive expression was abolished in *ein3 eil1* mutant (Solano et al., 1998; Zander et al.,
102 2012; Zhu et al., 2011). Given that EIN3 is a positive regulator of *ERF1* and *ORA59*, it was
103 assessed whether EIN3 and EIL1 control JA and ET synergy on defense gene expression. JAZ
104 proteins recruited histone deacetylase 6 (HDA6) as a corepressor to deacetylate histones and
105 interacted with EIN3/EIL1 to repress EIN3/EIL1-mediated transcription (Solano et al., 1998;
106 Zander et al., 2012; Zhu et al., 2011). JA led to JAZ degradation and removed JAZ-HDA6 from
107 EIN3/EIL1, and ET enhanced EIN3/EIL1 accumulation, enabling EIN3/EIL1 to converge JA
108 and ET signaling. The role as an integrative hub for JA and ET signaling has also been assigned
109 to subunits of the Mediator complex that connects transcription factors with the RNA
110 polymerase II machinery (Bäckström et al., 2007). The Mediator subunit MED25 physically
111 interacted with several transcription factors, including *ERF1*, *ORA59*, and EIN3/EIL1, and was
112 required for *ERF1*- and *ORA59*-activated *PDF1.2* expression (Çevik et al., 2012; Yang et al.,
113 2014). On the other hand, SA suppressed JA-dependent transcription by negatively affecting
114 *ORA59* protein abundance, suggesting that *ORA59* acts as a node for SA and JA antagonism
115 (He et al., 2017; Van der Does et al., 2013).

116 In this study, we report that the previously undefined *cis*-element ERELEE4 is critical for
117 JA/ET-induced transcription and is frequently present in the promoters of JA/ET-responsive
118 genes. In a yeast one-hybrid (Y1H) screening, *ORA59* was identified as a specific transcription
119 factor that binds to the ERELEE4 element, although *ORA59* was previously known to regulate
120 gene transcription by binding to the GCC box. Depending on whether plants are exposed to JA
121 or the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and ET, *ORA59* exhibited
122 preferential binding to the GCC box and ERELEE4, respectively. The present study explores

123 how two defense hormones JA and ET coordinate gene expression required for plant immunity
124 through the regulation of ORA59.

125

126 **Results**

127

128 **ERE is the *cis*-acting element for ET-responsive *GLIP1* expression**

129 We previously demonstrated that *Arabidopsis GDSL lipase 1 (GLIP1)* is an ET-responsive
130 defense gene that confers resistance to necrotrophic pathogens (Kim et al., 2014; Kim et al.,
131 2013; Kwon et al., 2009; Oh et al., 2005). In an expression analysis, Col-0 plants exhibited
132 strong *GLIP1* expression in response to ACC and ET, but a slight increase in *GLIP1* expression
133 upon methyl JA (MeJA) treatment (Supplementary Fig. 1a,b), which is in line with previous
134 results (Kim et al., 2013; Oh et al., 2005). However, both ACC- and MeJA-induced *GLIP1*
135 expression was abolished in ET-insensitive *ein2* and *ein3 eil1* and JA-insensitive *coil* mutants,
136 indicating that *GLIP1* induction requires both ET and JA signaling pathways (Supplementary
137 Fig. 1c,d). Whereas EIN2- and EIN3/EIL1-dependent *GLIP1* expression is consistent with our
138 previous observation (Kim et al., 2013), COI1 dependency may result from EIN3 regulation
139 by COI1-JAZ (Zhu et al., 2011).

140 To investigate how *GLIP1* expression is modulated by ET, the *GLIP1* promoter was
141 searched for the *cis*-element critical for ET-responsive *GLIP1* expression. In previous studies,
142 promoter analysis of ET- and JA-responsive *PR* genes led to the identification of the conserved
143 sequence AGCCGCC or GCC box that serves as a binding site for ERFs (Brown et al., 2003;
144 Hao et al., 1998; Ohme-Takagi and Shinshi, 1995). Accordingly, we expected the presence of
145 the GCC box in the *GLIP1* promoter and scanned the 2966-bp *GLIP1* promoter region
146 upstream of the transcription start site for *cis*-acting elements, using the PLACE program
147 (<http://www.dna.affrc.go.jp/PLACE/>). This analysis revealed that the *GLIP1* promoter has no

148 GCC box sequences and is enriched with binding motifs related to hormone and pathogen
149 responses, which include two ET-responsive elements, ERELEE4 (AWTTCAAA) and
150 RAV1AAT (CAACA) (Supplementary Table 1). ERELEE4 has been identified in promoter
151 regions of tomato *E4* and carnation *glutathione-S-transferase 1 (GST1)* genes, but poorly
152 characterized (Itzhaki et al., 1994; Montgomery et al., 1993). RAV1AAT has been isolated as
153 the binding motif for the *Arabidopsis* related to ABI3/VP1 1 (RAV1) transcription factor
154 belonging to the APETALA2/ERF superfamily (Kagaya et al., 1999). ERELEE4 was located
155 at 4 positions and RAV1AAT at 11 positions, here designated as ERE1 to ERE4 and RAV1 to
156 RAV11, respectively, upward from the transcription start site. In the case of EREs, there were
157 two different sequences, ATTTCAAA at ERE1, ERE3, and ERE4 and AATTCAAA at ERE2.

158 To examine whether ERE and RAV are key regulatory elements for ET-induced *GLIP1*
159 expression, we introduced the chimeric constructs of the *GLIP1* promoter (*pGLIP1*) and the β -
160 *glucuronidase (GUS)* reporter gene into *Arabidopsis* protoplasts and performed transient GUS
161 reporter assays. In accordance with previous results (Kim et al., 2013), *pGLIP1* elevated GUS
162 activity in response to ET and ACC, compared to mock treatments (Fig. 1a and Supplementary
163 Fig. 2a). *pGLIP1* constructs with a series of 5' deletions (*pGLIP1*-2466, -1466, -966, and -566)
164 were made and assayed for their ability to drive ET/ACC-induced GUS expression. As *pGLIP1*
165 became shorter and RAV and ERE elements were lost, GUS activity decreased proportionally.
166 No GUS activity was driven by *pGLIP1*-966 containing 3 RAV elements (RAV1 to RAV3).
167 The ability of ERE and RAV to respond to ET/ACC was further tested using synthetic
168 promoters, in which a minimal promoter (TATA-box) was fused to four tandem copies (4x) of
169 ERE and RAV and their mutated versions mERE and mRAV (Fig. 1b and Supplementary Fig.
170 2b). Among two ERE sequences in *pGLIP1*, more frequent ATTTCAAA was used for the
171 synthetic promoter. The 4xERE promoter strongly triggered ET/ACC-induced GUS expression
172 compared to 4xRAV, suggesting that ERE is critical for ET/ACC-mediated *GLIP1* expression.

173 Their mutated versions had little effect on GUS expression. Next, *pGLIP1*-mediated GUS
174 activity was measured after mutation of one by one or 4 EREs at once (Fig. 1c). *pGLIP1* with
175 individual ERE mutations displayed significantly decreased GUS activity (47-69% reduction)
176 compared to the native promoter. GUS activity driven by *pGLIP1^{mEREs}* with all 4 EREs mutated
177 was largely eliminated. *pGLIP1* activity was further assessed in Col-0 plants harboring
178 *pGLIP1:GUS* or *pGLIP1^{mEREs}:GUS* reporters. Histochemical staining developed strong GUS
179 signals in *pGLIP1:GUS* plants upon ACC treatment and in response to *B. cinerea* infection,
180 which were largely abolished in *pGLIP1^{mEREs}:GUS* plants (Fig. 1d). These results together
181 demonstrate that ERE plays a major role in ET-responsive *GLIP1* expression.

182 The requirement of ERE elements for *GLIP1* expression was additionally assessed by
183 generating transgenic plants, *pGLIP1:GLIP1-GFP* and *pGLIP1^{mEREs}:GLIP1-GFP*, which
184 express GLIP1 fused to green fluorescent protein (GFP) at the C-terminus under the control of
185 *pGLIP1* and *pGLIP1^{mEREs}*, respectively, in the *glip1* mutant background. First, plants were
186 infected with *A. brassicicola*, and examined for disease development. Whereas *glip1* mutant
187 was highly susceptible to *A. brassicicola*, *pGLIP1*-driven *GLIP1-GFP* expression restored
188 disease resistance in *glip1* (Fig. 2a-c). Consistently, *GLIP1-GFP* transcripts and GLIP1-GFP
189 proteins accumulated and GFP fluorescence was detected in *pGLIP1:GLIP1-GFP* plants, but
190 not in *pGLIP1^{mEREs}:GLIP1-GFP* plants, in response to *A. brassicicola* and ACC treatments (Fig.
191 2d-f). These results together indicate that ERE elements are essential for *GLIP1* expression
192 during the immune response.

193

194 **ORA59 is an ERE-binding transcription factor**

195 Next, we searched for a transcription factor(s) that regulate ET-responsive *GLIP1* expression,
196 and for this, performed Y1H screening using the ERE sequence ATTTCAA as bait. The yeast
197 strain, harboring three tandem copies of ERE fused to *HIS3* and *lacZ* genes, was transformed

198 with a prey library composed of 1050 *Arabidopsis* transcription factor cDNAs (Welchen et al.,
199 2009). Screening of 2×10^6 transformants yielded 84 positive clones growing on selective
200 media lacking histidine and containing 3-amino-1,2,4-triazole (3-AT) (Supplementary Table 2).
201 Among these positive clones, ORA59, related to AP2.2 (RAP2.2), and caprice-like MYB3
202 (CPL3) were subjected to further analysis, as they most strongly increased β -galactosidase
203 reporter activity. Re-transformation with the recovered plasmid DNAs enabled yeast cells to
204 grow on selective media (Fig. 3a).

205 To test for *in vitro* binding of ORA59, RAP2.2, and CPL3 to the ERE element, we
206 performed an electrophoretic mobility shift assay (EMSA) using recombinant proteins and
207 DNA probes of two ERE sequences ATTTCAAA and AATTCAAA (Fig. 3b,c). Whereas
208 ORA59 formed a shifted band, RAP2.2 and CPL3 exhibited weak binding. ORA59 had similar
209 levels of binding activity to these two ERE sequences. The addition of excess amounts of
210 unlabeled ERE probes effectively competed the binding, verifying specific ORA59 binding to
211 the ERE sequences (Fig. 3d). Transient GUS reporter assays were then performed to determine
212 whether they can induce transcription through the ERE *in vivo* (Fig. 3e). The *pGLIP1* and
213 synthetic 4xERE and 4xRAV promoters, and their mutant versions *pGLIP1^{mEREs}*, 4xmERE, and
214 4xmRAV were used as reporter constructs, and together with effector constructs of CPL3,
215 RAP2.2, and ORA59, were transformed into *Arabidopsis* protoplasts. Whereas ORA59
216 strongly activated *pGLIP1*- and 4xERE-mediated GUS expression, slight GUS expression
217 driven by both *pGLIP1* and *pGLIP1^{mEREs}* was observed with RAP2.2, and no GUS activity was
218 observed with CPL3. Transactivation by ORA59 was dependent on the ERE in reporter genes,
219 because no activity was detected for reporters with *pGLIP1^{mEREs}* and 4xmERE or with 4xRAV
220 and 4xmRAV. These results suggest that ORA59 controls *GLIP1* expression via ERE binding.

221 Because the GCC box has been determined as a specific binding site for ORA59 and other
222 ERFs in previous reports (Hao et al., 1998; Ohme-Takagi and Shinshi, 1995; Zarei et al., 2011),

223 the binding activity of ORA59 to GCC box and ERE was compared using recombinant ORA59
224 proteins. In addition to full-length ORA59, several truncated forms of ORA59 were prepared
225 (Fig. 4a-c). In the EMSA analysis, full-length ORA59 bound to GCC box more strongly than
226 to ERE (Fig. 4d). Noticeably, N-terminal deletion (F1) dramatically enhanced ORA59 binding
227 to GCC box, but rather abrogated ERE-binding activity. In contrast, ORA59 with partial C-
228 terminal deletion (F3) showed much stronger ERE binding. We failed to secure soluble ORA59
229 proteins with deletion of the entire C-terminal region after the AP2 domain. The C-terminal
230 (F2) and N-terminal (F4) portions alone exhibited no DNA binding activities, as expected for
231 ORA59 without the DNA-binding AP2 domain. AP2 domain alone had stronger binding
232 activity to ERE than to GCC box, which was reversed for full-length ORA59. These results
233 suggest that ORA59 may form distinct structural conformations in binding to ERE and GCC
234 box, and the N-terminal and C-terminal portions of ORA59 affect ORA59 binding to ERE and
235 GCC box in different manners. The N-terminal portion may have a positive or negative effect
236 on ORA59 binding to ERE and GCC box, respectively, and this may be the opposite for the C-
237 terminal portion.

238

239 **ORA59 binding to ERE and GCC box is differentially regulated in ET and JA signaling**

240 To study how ORA59 interacts with ERE and GCC box elements *in vivo*, we additionally
241 prepared transgenic plants (*35S:ORA59-GFP*) overexpressing ORA59 fused to GFP at the C-
242 terminus under the control of the CaMV 35S promoter (Supplementary Fig. 3a,b). As observed
243 in a previous study (Pré et al., 2008b), *35S:ORA59-GFP* plants showed a dwarf phenotype.
244 Basal transcript levels of *GLI1* were increased in *35S:ORA59-GFP* plants, and ACC- and *B.*
245 *cinerea*-induced *GLI1* expression was diminished in *ora59* mutant, confirming that ORA59
246 is the key regulator of *GLI1* expression (Supplementary Fig. 3c). *35S:ORA59-GFP* plants
247 exhibited enhanced resistance against *B. cinerea*, as determined by lesion size and abundance

248 of fungal actin gene (Supplementary Fig. 3d). In contrast, susceptibility to *B. cinerea* was
249 increased in *ora59* plants. Among independent lines, *35S:ORA59-GFP* (#6) was used for
250 further study.

251 ORA59-GFP protein levels were monitored in *35S:ORA59-GFP* plants exposed to ACC
252 and MeJA. ORA59-GFP proteins rapidly disappeared in the presence of the protein synthesis
253 inhibitor cycloheximide (CHX), which was repressed by treatment with the proteasome
254 inhibitor MG132 (Fig. 5a). ORA59-GFP protein abundance was elevated in ACC- or MeJA-
255 treated *35S:ORA59-GFP* plants. These results indicate that ORA59 undergoes 26S
256 proteasome-dependent degradation, and ET and JA enhance the stability of ORA59 proteins.

257 To examine ORA59 binding to ERE and GCC box *in planta*, nuclear extracts were
258 prepared from ACC/ET- and MeJA-treated *35S:ORA59-GFP* plants and assessed for binding
259 to these elements by EMSA. Noticeably, nuclear extracts from ACC/ET- and MeJA-treated
260 *35S:ORA59-GFP* plants showed DNA binding activities with differential preference for ERE
261 and GCC box, respectively, which were largely diminished in Col-0 and *ora59* extracts (Fig.
262 5b,c and Supplementary Fig. 4). These results imply that DNA-protein complexes observed are
263 mostly of ORA59 expressed in *35S:ORA59-GFP* plants. Col-0 nuclear extracts, albeit weakly
264 binding, retained the hormone-dependent preference for ERE and GCC box.

265 The Ser-rich sequence of ORA59 (Fig. 4a) led us to speculate that DNA binding properties
266 of ORA59 may be regulated by post-translational modifications, such as phosphorylation. To
267 address this, nuclear extracts of *35S:ORA59-GFP* plants were immunoprecipitated with an
268 anti-GFP antibody, and the isolated proteins were subjected to Western blotting with an anti-
269 phospho-Ser/Thr antibody. It revealed that ORA59 is phosphorylated in ACC- and MeJA-
270 treated plants (Fig. 5b). To further verify this, *35S:ORA59-GFP* nuclear extracts were treated
271 with lambda phosphatase before incubation with DNA probes. Phosphatase treatment led to
272 dephosphorylation of ORA59, which was accompanied by a significant reduction in ORA59

273 binding to ERE and GCC box, and particularly, lack of hormone-dependent binding sequence
274 specificity (Fig. 5d). When accumulated after MG132 treatment, ORA59-GFP proteins showed
275 similar results with much lower level of phosphorylation, compared to those treated with ACC
276 and MeJA (Fig. 5e). This indicates that ORA59 is normally phosphorylated to a certain extent
277 and the phosphorylation level is increased in response to ET and JA. These results suggest that
278 ET- and JA-mediated ORA59 phosphorylation is critical for ORA59 activity.

279 Considering the role of ORA59 in the ET-JA crosstalk, the next question was how ORA59
280 binds to ERE and GCC box when activated by two hormones simultaneously. To investigate
281 this, *35S:ORA59-GFP* plants were co-treated with ACC and JA, and then subjected to EMSA.
282 A combination of ACC and JA did not further increase DNA binding of ORA59, nor did it
283 change ORA59 protein abundance, compared to treatment with each hormone (Fig. 5f). In
284 contrast, the level of ORA59 phosphorylation was largely increased by ACC and MeJA co-
285 treatments. We then examined whether hormone-dependent DNA binding properties of ORA59
286 are correlated with transcriptional activity. GUS reporter assays were conducted using 4xERE
287 and 4xGCC box synthetic promoters. In Col-0 protoplasts, ACC treatment induced a large
288 increase in transcription through the ERE but a less increase through the GCC box (Fig. 5g).
289 Conversely, a large increase was observed with the GCC box but a modest increase with the
290 ERE in response to MeJA treatment. Simultaneous treatments with ACC and MeJA led to a
291 synergistic increase in GUS activity through both ERE and GCC box. This transcriptional
292 activation was not observed with mutated elements and significantly decreased in *ora59*
293 protoplasts. These results suggest that ET- and JA-regulated transcription is associated with
294 differential DNA binding of ORA59, and ORA59 regulates ET and JA synergy at the level of
295 transcriptional activation, but not at the level of DNA binding.

296

297 **ORA59 regulates gene expression by direct binding to ERE and GCC box**

298 It was then determined whether other genes are also regulated by ORA59 in ET/JA-dependent
299 ways. We found that *PDF1.2* genes have different distributions of ERE and GCC box in their
300 promoters such that promoters of *PDF1.2a*, *PDF1.2b*, and *PDF1.2c* have a single GCC box,
301 one GCC box and two ERE, and a single ERE elements, respectively (Fig. 6a). We conducted
302 GUS reporter assays using *PDF1.2* promoters. Whereas GUS expression driven by *PDF1.2*
303 promoters was induced by both ACC and MeJA, *PDF1.2a* with only GCC box and *PDF1.2c*
304 with only ERE responded more strongly to MeJA and ACC, respectively, than to the other (Fig.
305 6b). Likewise, mutations of respective elements in *PDF1.2* promoters largely affected
306 transcriptional activation, and in particular, mutation of either ERE or GCC box in the *PDF1.2b*
307 promoter containing both elements more significantly reduced ACC- or MeJA-induced
308 transcription, respectively. Gene expression analysis in Col-0 plants showed that endogenous
309 transcript levels of *PDF1.2* genes were increased by ACC and MeJA treatments with similar
310 preference for hormones observed in GUS reporter assays, and this increase was abolished in
311 *ora59* plants (Fig. 6c).

312 Given that ORA59 binds to ERE and GCC box in EMSA, we performed chromatin
313 immunoprecipitation (ChIP)-qPCR analysis to examine whether ERE- and GCC box-driven
314 transcriptional activation is induced through direct ORA59 binding to these elements in the
315 *GLI1* and *PDF1.2* promoters. *35S:ORA59-GFP* plants were treated with ACC and MeJA, and
316 their extracts were used for precipitating ORA59-bound DNA fragments with an anti-GFP
317 antibody. In the *GLI1* promoter, all four ERE-containing fragments were enriched in ORA59
318 binding, and this enrichment was increased more significantly with ACC treatment than with
319 MeJA (Fig. 6d). In addition, ORA59 binding was greatly enriched at ERE and GCC box sites
320 of the *PDF1.2a*, *PDF1.2b*, and *PDF1.2c* promoters in response to ACC and MeJA treatments,
321 respectively, which was consistent with the results of transcriptional activation (Fig. 6e). No
322 binding of ORA59 was observed in negative control fragments without ERE and GCC box

323 sequences. These results indicate that ORA59 regulates ET- and JA-responsive gene expression
324 by binding to ERE and GCC box directly and with hormone-dependent differential preference.

325

326 **Identification of ORA59-regulated ET- and JA-responsive genes by RNA-seq analysis**

327 Based on differential responses of ORA59 to ACC and MeJA in gene regulation, we speculated
328 that ORA59 may regulate distinct gene sets in the ET and JA pathways. Therefore, to identify
329 ET- and JA-responsive ORA59 downstream genes, we performed RNA-sequencing (RNA-seq)
330 analysis using biological replicates of ACC- and MeJA-treated Col-0 and *ora59* plants
331 (Supplementary Table 3). Differentially expressed genes (DEGs) between mock (water) and
332 ACC/MeJA treatments were selected in Col-0 and *ora59* plants based on the cutoff (adjusted
333 P (P_{adj}) < 0.05 , \log_2 fold change ($|\log_2 FC| \geq 1$). Col-0 had far more DEGs than *ora59*
334 mutant, showing that 516 and 105 genes were differentially expressed in ACC-treated Col-0
335 and *ora59*, and 683 and 134 genes in MeJA-treated Col-0 and *ora59*, respectively (Fig. 7a).
336 This implies that ORA59 is an essential regulator of gene expression in ET and JA responses.
337 Considering that DEGs in *ora59* mutant are ORA59-independent, among 516 and 683 DEGs
338 in ACC- and MeJA-treated Col-0, after subtracting 37 and 87 genes co-regulated in Col-0 and
339 *ora59*, 479 and 596 genes were defined as ACC- and MeJA-responsive ORA59-regulated
340 genes, respectively (Fig. 7b). It was noted that the majority of ORA59-dependent genes were
341 upregulated in response to ACC (346 out of 479, 72.2%) and in response to MeJA (443 out of
342 596, 74.3%), suggesting that ORA59 primarily functions as a transcriptional activator of gene
343 expression. The overlap between ACC- and MeJA-responsive ORA59-regulated genes was
344 relatively small, and only 54 genes were shared (Supplementary Fig. 5).

345 We then performed Gene Ontology (GO) enrichment analysis of ORA59-regulated DEGs,
346 using GO Biological Process (BP) terms provided by PANTHER database
347 (<http://geneontology.org>). (Supplementary Table 4). The analysis revealed that ACC-

348 responsive DEGs are enriched in responses to stress, oxygen-containing compounds, stimulus,
349 and oxygen levels GO BP terms, while MeJA-responsive DEGs are enriched in metabolic
350 processes of organic acids, S-glycosides, and secondary metabolites, and in responses to stress
351 and chemicals GO BP terms (Fig. 7c). Enriched GO BP terms indicate that ACC and MeJA
352 regulate distinct biological processes in an ORA59 dependent manner, only co-regulating the
353 ‘response to stress’. We then determined the occurrence and enrichment of ERE and GCC box
354 in promoters of ORA59-regulated ACC- and MeJA-responsive genes, compared to whole
355 *Arabidopsis* 34362 genes. Noticeably, ERE was present at a much higher frequency (28.5%)
356 than GCC box (4.4%) in whole gene promoters (Fig. 7d). Statistically significant enrichment
357 of ERE was observed in both ACC (Fisher exact test $P = 3.38 \times 10^{-8}$)- and MeJA (Fisher exact
358 test $P = 2.12 \times 10^{-8}$)-responsive genes, but GCC box was only enriched in MeJA (Fisher exact
359 test $P = 3.98 \times 10^{-3}$)-responsive genes.

360

361 **Identification of ORA59 target genes involved in disease resistance**

362 For further functional analysis, we focused primarily on genes whose expression was increased
363 by ACC and MeJA treatments. Among ACC- and MeJA-responsive DEGs, 63 ($|\log_2 \text{FC}| \geq$
364 2) and 55 ($|\log_2 \text{FC}| \geq 3$) upregulated genes were selected from the five most significantly
365 enriched GO BP terms, respectively, and their expression was validated by RT-qPCR analysis
366 (Supplementary Table 5). Towards isolating ORA59 target genes involved in the immune
367 response, the expression of selected genes was assessed in *35S:ORA59-GFP* and *B. cinerea*-
368 treated Col-0 plants, among which ACC-responsive eight ($|\log_2 \text{FC}| \geq 4$) and MeJA-
369 responsive seven ($|\log_2 \text{FC}| \geq 4$) genes were chosen to further investigate their functions in
370 disease resistance (Supplementary Fig. 6 and Fig. 8a).

371 We performed tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) in
372 *Arabidopsis* Col-0 (Ahn et al., 2015; Burch-Smith et al., 2006). TRV2 vector (control) and

373 VIGS constructs carrying DNA fragments of 15 genes were transformed into *Agrobacterium*,
374 which was followed by infiltration into true leaves of seedlings. Phenotypes of VIGS plants
375 and transcript levels of target genes were determined in rosette leaves at 19 to 21 days post-
376 infiltration (dpi). Consistent with previous observations (Burch-Smith et al., 2006), silencing
377 of *phytoene desaturase* (*PDS*), used as a marker of VIGS, caused photo-bleaching of leaves
378 and reduction of *PDS* expression compared to the TRV2 control (Supplementary Fig. 7). All
379 VIGS plants were successfully generated with more than 90% reduction in gene expression,
380 compared to the TRV2 control, and therefore challenged with *B. cinerea* (Fig. 8b). Disease
381 development, lesion size, and abundance of fungal actin gene were determined in the infected
382 leaves (Fig. 8c-e). It was observed that susceptibility is increased by VIGS of 10 genes, i.e.,
383 ACC-responsive 6 and MeJA-responsive 4 genes, encoding ERF1, rotamase FKBP 2
384 (ROF2)/FK506-binding protein 65 (FKBP65), UDP-glucose/galactose transporter 1 (UTR1),
385 beta glucosidase 30 (BGLU30)/dark inducible 2 (DIN2), N-acetyltransferase activity 1
386 (NATA1), unusual serine protease inhibitor (UPI), cytochrome P450, family 81, subfamily F,
387 polypeptide 4 (CYP81F4), drought-repressed 4 (DR4), ERF016, and myb domain protein 113
388 (MYB113). Consistently, the expression of these genes was significantly reduced in *ora59*
389 plants compared to Co-0, in response to *B. cinerea* infection, suggesting that they function
390 downstream of ORA59 in disease resistance (Supplementary Fig. 8). Five other genes
391 displayed no altered responses to *B. cinerea* after VIGS, perhaps because they are not related
392 to disease resistance or it is attributed to genetic redundancy.

393 The isolated ORA59 target genes were scanned for the presence of ERE and GCC box in
394 their promoters. Whereas 3 out of 10 genes had none of these elements, the other ACC- and
395 MeJA-responsive genes contained one or more ERE and/or GCC box elements in the promoters
396 (Fig. 8f). We carried out CHIP-qPCR analyses using *35S:ORA59-GFP* plants to determine
397 whether ERE- and GCC box-containing genes are regulated by direct binding of ORA59 to

398 their promoters. ChIP assays showed that ORA59 binding is enriched at ERE- and GCC box
399 motifs of the promoters, indicating that they are direct targets of ORA59. These results suggest
400 that ORA59 modulates immune responses to necrotrophic pathogens through regulation of
401 direct and indirect target genes with diverse activities (Fig. 8g).

402

403 **Discussion**

404

405 Phytohormone signaling and crosstalk are critical for regulating plant immune responses. In
406 particular, JA and ET have been identified as defense signals required for resistance against
407 necrotrophic pathogens (Dong, 1998; Pieterse et al., 2009; Zhu, 2014). Upon pathogen
408 infection, JA and ET are synthesized rapidly and they work together, forming signaling
409 networks which involve interactions among signaling components (Koornneef and Pieterse,
410 2008; Yang et al., 2015). Given that hormone signaling evokes output responses through gene
411 regulation, JA and ET induce the expression of defense genes, such as *PDFI.2*, in a synergistic
412 and interdependent manner (Koornneef and Pieterse, 2008; Thomma et al., 1998; Thomma et
413 al., 1999). In this context, we observed that there is an overlap between JA- and ET-responsive
414 genes, but on the other hand, other subsets of genes were differentially regulated by JA and ET,
415 which was also described previously (Schenk et al., 2000).

416 JA/ET-mediated gene transcription typically occurs through the action of ERFs, among
417 which ERF1 and ORA59 regulate *PDFI.2* expression by binding to the GCC box and have
418 been considered as integrators of JA and ET signaling (Lorenzo et al., 2003; Pré et al., 2008a;
419 Zarei et al., 2011). While ERF1 and ORA59 have been shown to regulate gene expression
420 commonly in JA and ET pathways, questions are raised about how they respond differently to
421 each hormone to induce JA- and ET-specific gene expression. In this study, we identified the
422 poorly characterized ERE as an ORA59-binding *cis*-element in addition to the GCC box.

423 EMSA, GUS reporter assays, and ChIP-qPCR analysis demonstrated that JA and ET enhance
424 protein stability, and DNA binding and transactivation activities of ORA59 with differential
425 preference for GCC box and ERE, respectively. While supporting this, ORA59 regulated genes
426 of different functional categories in JA and ET pathways, as shown by RNA-seq and GO
427 analysis. Our results provide insights into the molecular basis of how JA and ET modulate
428 ORA59 to cooperatively and differentially regulate gene expression and to accomplish the fine-
429 tuning of immune responses (Fig. 8g).

430 EIN3 functions as a positive regulator of *ERF1* and *ORA59* expression (Solano et al., 1998;
431 Zander et al., 2012; Zhu et al., 2011). JA- and ET-mediated induction of *ERF1* and *ORA59* was
432 abolished in *ein3 eil1* mutant, and *ORA59* promoter activity was increased by *EIN3*
433 overexpression. In addition, EIN3 directly bound to the *ERF1* promoter, indicating that *ERF1*
434 is the target gene of EIN3. Two different nucleotide sequences, TACAT and TTCAAA, have
435 been identified as the EIN3-binding site (EBS) in the promoters of several EIN3-regulated
436 genes, such as *ERF1*, *EBF2*, *protochlorophyllide oxidoreductase A and B (PORA/B)*, *hookless*
437 *1 (HLS1)*, and *microRNA164 (miR164)* (An et al., 2012; Huang et al., 2020; Konishi and
438 Yanagisawa, 2008; Li et al., 2013; Solano et al., 1998; Zhong et al., 2009), and here they will
439 be referred to as EBS1 and EBS2, respectively. In the course of this work, we have recognized
440 that the EBS2, TTCAAA, is part of the ERE sequence AWTTCAAA, and this is especially true
441 for the EBS2 in *PORB* and *HLS1* promoters. Therefore, we wonder whether EIN3-regulated
442 genes containing the EBS2 in their promoters, which overlaps with the ERE, are also targets
443 of ORA59, and whether ORA59 is implicated in other cellular processes, such as light signaling
444 and seedling development. It may be possible that EIN3 and ORA59 share and co-regulate
445 certain target genes, which is supported by the evidence that EIN3 and ORA59 proteins interact
446 together (He et al., 2017).

447 In our RNA-seq analysis, *ERF1* was isolated as an ORA59-regulated ET-responsive gene.

448 In addition to the previously identified EBS2 (Solano et al., 1998), the *ERF1* promoter has a
449 separate ERE, to which *ORA59* directly bound as determined by ChIP analysis, implying that
450 *ORA59* is the upstream regulator of *ERF1*. Conversely, a previous study showed that *ORA59*
451 expression is largely increased in *ERF1*-overexpressing plants (Van der Does et al., 2013).
452 Given that the *ORA59* promoter contains both ERE and GCC box, *ORA59* and *ERF1* may
453 activate each other via a positive feedback loop. Furthermore, *ERF1* bound to another stress-
454 responsive element DRE/CRT during abiotic stress responses, as shown in a previous study
455 (Cheng et al., 2013). This and our data suggest that ERFs, including *ERF1* and *ORA59*, may
456 bind to distinct types of *cis*-elements, depending on hormone and stress stimuli. On the other
457 hand, studies have shown that other transcription factors, *TGA2/4/6* (class II TGAs) and
458 *WRKY33*, positively regulate *ORA59* expression in response to ACC and *B. cinerea* infection
459 through binding to the TGA binding site TGACGT and the W-box TTGAC(C/T) in the *ORA59*
460 promoter, respectively (Birkenbihl et al., 2012; Zander et al., 2014). Further investigation is
461 needed on how ET/JA-regulated EIN3, *ORA59*, and *ERF1*, and other types of transcription
462 factors, such as TGAs and WRKYs, interact and coordinately regulate gene expression in the
463 transcriptional and protein interaction networks.

464 Protein phosphorylation regulates the function of transcription factors by modulating DNA
465 binding, transcriptional activity, protein stability, cellular localization, and protein-protein
466 interactions. Many reports provide evidence that ERFs are regulated by phosphorylation
467 (Huang et al., 2016; Licausi et al., 2013; Phukan et al., 2017). Phosphorylation of the tomato
468 ERF Pti4 by Pto kinase enhanced Pti4 binding to the GCC box, increasing the expression of
469 GCC box-containing *PR* genes (Gu et al., 2000). Mitogen-activated protein kinase
470 (MAPK/MPK) cascades have been implicated in ERF phosphorylation. When phosphorylated
471 by blast and wound-induced MAPK1 (BWMK1), the rice ET-responsive element-binding
472 protein 1 (OsEREBP1) showed enhanced DNA binding activity to the GCC box, and

473 concomitantly, the increased GCC box-driven transcription (Cheong et al., 2003).
474 Transactivation by the tobacco NtERF221 (originally designated as ORC1) was positively
475 affected by a MAPK kinase, JA-factor stimulating MAPKK1 (JAM1) (De Boer et al., 2011).
476 The *Arabidopsis* ERF6 served as an MPK substrate, and its protein stability and nuclear
477 localization were increased by MPK3/MPK6-mediated ERF6 phosphorylation (Meng et al.,
478 2013; Wang et al., 2013). In this study, we showed that ORA59 phosphorylation is elevated in
479 plants treated with either ACC or MeJA. ORA59 activated through ACC and JA signals had
480 differential preferences for ERE and GCC box, in addition to enhanced DNA binding, which
481 was eliminated by phosphatase-mediated dephosphorylation of ORA59. Likewise, ORA59
482 proteins which accumulated in MG132-treated plants showed a similar level of binding to these
483 elements. These results suggest that phosphorylation regulates both affinity and specificity of
484 ORA59 for DNA sequences. Furthermore, recombinant ORA59 proteins with deletion of the
485 N- and C-terminal parts showed differential GCC box- and ERE-binding activities, suggesting
486 that ORA59 may form distinct structures with different affinities for ERE and GCC box, and
487 this may be regulated by hormone-dependent phosphorylation events (Fig. 8g). Therefore, it is
488 important to investigate whether ORA59 phosphorylation occurs in ET- and JA-dependent
489 ways, and whether it modulates the structure and activity of ORA59. A combination of ACC
490 and MeJA treatments further increased the level of phosphorylation, but not that of DNA
491 binding activity of ORA59, suggesting that ORA59 phosphorylation may be involved in ET
492 and JA synergy at the level of transcriptional activation, e.g., interaction with other
493 cofactors/transcription factors and transcription machinery components.

494 Gene expression, VIGS, and ChIP analysis led to the identification of direct target genes
495 of ORA59, *ERF1*, *ROF2/FKBP65*, *UTR1*, *BGLU30/DIN2* as ACC-responsive genes, and
496 *CYP81F4*, *DR4*, and *ERF016* as MeJA-responsive genes, and indirect target genes, *NATA1*,
497 *UPI*, and *MYB113*. These ORA59 target genes are clustered into four functional groups. First,

498 *ERF1*, *ERF016*, and *MYB113* encode transcription factors, which are involved in the regulation
499 of defense gene expression. *ERF1* has been well characterized to enhance *PDF1.2* expression
500 and disease resistance (Zarei et al., 2011). *ERF016* bound to the GCC box of the *PDF1.2*
501 promoter and *erf016* mutants displayed a significant increase in susceptibility to *B. cinerea*
502 (Hickman et al., 2017; Ou et al., 2011). *MYB113* expression was much reduced in *ora59*
503 mutants, suggesting that *MYB113* functions downstream of *ORA59* (Zander et al., 2014).
504 Second, *DR4* and *UPI* encode protease inhibitors implicated in resistance to necrotrophic fungi
505 (Brodersen et al., 2006; Gosti et al., 1995; Laluk and Mengiste, 2011). Third, *BGLU30/DIN2*,
506 *CYP81F4*, and *NATA1* function in secondary metabolic pathways. *BGLU30/DIN2*, encoding a
507 β -glucosidase, and *CYP81F4*, encoding a cytochrome P450 monooxygenase, showed activities
508 associated with glucosinolate metabolism (Morikawa-Ichinose et al., 2020; Pfalz et al., 2011;
509 Zhang et al., 2020). Glucosinolates and their breakdown products function in defense against
510 pathogens (Bednarek, 2012), supporting the possibility that *BGLU30* and *CYP81F4* may play
511 a role in plant immunity. *NATA1* was identified as an acetyltransferase that acetylates ornithine
512 and putrescine in response to coronatine, JA, and *P. syringae* infection (Adio et al., 2011; Lou
513 et al., 2016). Fourth, *ROF2/FKBP65*, encoding a peptidyl-prolyl cis-trans isomerase, and *UTR1*,
514 encoding a nucleotide sugar transporter, are involved in protein folding and endoplasmic
515 reticulum (ER) quality control processes. Knockout or overexpression of *ROF2/FKBP65*
516 decreased or increased resistance against *P. syringae*, respectively (Pogorelko et al., 2014).
517 *UTR1*, required for the transport of UDP-glucose into the ER, may be involved in plant
518 immunity, because proper folding of immune receptors and PRRs relies on the ER quality
519 control system (Eichmann and Schäfer, 2012; Reyes et al., 2006). Further studies on the
520 functions of *ORA59* target genes will improve our understanding of the ET-JA signaling
521 network and involving components in the regulation of plant immunity.

522

523 **Methods**

524

525 **Plant materials and growth conditions**

526 *Arabidopsis thaliana* (ecotype Columbia, Col-0) plants were grown at 23°C under long-day
527 conditions in a 16-h light/8-h dark cycle. The mutant lines used in this study are *glip1-1* (Oh et
528 al., 2005), *ein2-1* (Roman and Ecker, 1995), *ein3-1eill-1* (Alonso et al., 2003), *ora59*
529 (CS_405772), and *coil* (SALK_095916). Homozygous lines were selected by PCR and
530 sequence analysis using gene-specific primers (Supplementary Table 6). To generate
531 *35S:ORA59-GFP* plants, the *ORA59* coding region was cloned into the pCHF3-GFP binary
532 vector under the control of the CaMV 35S promoter. To generate *pGLIP1:GUS* and
533 *pGLIP1^{mEREs}:GUS* plants, the *GLIP1* promoter region (-1 to -2966 bp) was amplified from
534 *Arabidopsis* gDNA by PCR and cloned into the pBI121 vector containing a *GUS* gene. ERE
535 mutations in the *GLIP1* promoter were generated by site-directed mutagenesis using primers
536 in Supplementary Table 6. For *pGLIP1:GLIP1-GFP* and *pGLIP1^{mEREs}:GLIP1-GFP* plants, the
537 *GLIP1* coding region was cloned into the pCAMBIA1300 vector containing a *GFP* gene, and
538 then *pGLIP1* and *pGLIP1^{mEREs}* were inserted upstream of *GLIP1-GFP* in the pCAMBIA1300
539 vector. The constructs were transformed into *Agrobacterium tumefaciens* GV3101 and then
540 introduced into Col-0 and *glip1-1* plants using the floral dip method (Clough and Bent, 1998).

541

542 **Plant treatments**

543 For pathogen infection, *B. cinerea* and *A. brassicicola* were grown on potato dextrose agar
544 plates for 2 weeks, and their spores were harvested and incubated in half-strength potato
545 dextrose broth for 2 h prior to inoculation as previously described (Broekaert et al., 1990). Six-
546 week-old leaves were inoculated with 5 µl droplets of spore suspensions (5×10^5 spores ml⁻¹).
547 Fungal growth was assessed by qPCR for the abundance of *A. brassicicola cutinase A*

548 (*AbCUTA*) and *B. cinerea actin* (*BcACT*) genes relative to *Arabidopsis tubulin 2* (*AtTU2*).
549 Lesion size was determined by measuring the diameter of the necrotic area. For chemical
550 treatments, 6-week-old plants were sprayed with 0.01% Silwet L-77 containing 1 mM SA, 1
551 mM ACC, 100 μ M MeJA, 50 μ M MG132, and 100 μ M CHX or incubated with 10 ppm ET in
552 hydrocarbon-free air. The treated plants were maintained at 100% humidity for the indicated
553 times.

554

555 **Transient expression assays**

556 For transient assays in *Arabidopsis* protoplasts, effector and reporter constructs were generated.
557 For effector constructs, coding regions of *ORA59*, *RAP2.2*, and *CPL3* were amplified from the
558 *Arabidopsis* cDNA library by PCR and cloned into the pUC18 vector for the expression of
559 hemagglutinin (HA)-tagged proteins in protoplasts (Cho and Yoo, 2011). For gene promoter-
560 reporter constructs, promoter regions of *GLIP1*, *PDF1.2a*, *PDF1.2b*, and *PDF1.2c* were
561 amplified from *Arabidopsis* gDNA by PCR and cloned into the pBI221 vector containing a
562 *GUS* gene. Mutations of ERE and GCC box elements in the promoter regions were generated
563 by site-directed mutagenesis using primers in Supplementary Table 6. For synthetic promoter-
564 reporter constructs, four copies of the native ERE or GCC box and four copies of respective
565 mutated versions were fused with the minimal *GLIP1* promoter (-1 to -122 bp) and cloned into
566 the pBI221 vector containing a *GUS* gene. *Arabidopsis* mesophyll protoplasts were isolated
567 and transfected as previously described (Yoo et al., 2007). Isolated protoplasts (2×10^4) were
568 transfected with a reporter DNA (20 μ g) alone or together with an effector DNA (20 μ g). GUS
569 activity was measured fluorometrically using 4-methylumbelliferyl- β -D-glucuronide as
570 substrate. The firefly luciferase (LUC) expressed under the control of the CaMV 35S promoter
571 was used as an internal control, and the activity was measured using the luciferase assay system
572 (Promega). Relative GUS activities were normalized with respect to the LUC activity.

573

574 **Histochemical GUS staining**

575 GUS staining was performed as previously described (Lee et al., 2017a). Rosette leaves were
576 incubated in a staining buffer (50 mM sodium phosphate, pH 7.0, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM
577 $K_4Fe(CN)_6$, 10 mM EDTA, and 0.2% Triton X-100) containing 4 mM 5-bromo-4-chloro-3-
578 indolyl- b-D-glucuronide (X-Gluc) for 16 h at 37°C. Stained leaves were cleared by several
579 washes with 70% ethanol.

580

581 **Yeast one-hybrid (Y1H) screening**

582 Y1H screening was performed as previously described (Welchen et al., 2009). To obtain a yeast
583 strain carrying the ERE sequence in front of the *HIS3* reporter gene, three tandem repeats of
584 the ERE were cloned into the pHIS3-NX vector, and the 3xERE-*HIS3* cassette was cloned into
585 the pINT vector, which confers resistance to the antibiotic G418. The clone in pINT1 was
586 introduced into the yeast strain Y187. Alternatively, the 3xERE was placed in front of the *lacZ*
587 reporter gene contained in the pLacZi vector (Clontech). Transcription factors interacting with
588 the ERE sequence were identified using a DNA library carrying a 1050 *Arabidopsis*
589 transcription factor ORFeome collection in the prey vector pDEST22 (Invitrogen). For Y1H
590 screening, plasmid DNA from the library (10 μ g) was introduced into yeast and a total of 2 x
591 10^6 transformants were plated on SD-Trp-His medium containing 0.2 mM 3-AT. The resulting
592 putative positive clones were streaked on fresh SD-Trp-His + 0.2 mM 3-AT medium to purify
593 colonies. The plasmid DNAs containing ORFs were rescued and retransformed into yeast for
594 confirmation.

595

596 **Protein expression and purification**

597 The full-length coding regions of *ORA59*, *RAP2.2*, *CPL3*, and the truncated regions of *ORA59*

598 were PCR-amplified using gene-specific primers (Supplementary Table 6). The PCR products
599 were cloned into the pMAL-x2X vector to generate proteins fused to the N-terminal maltose-
600 binding protein (MBP) and His-tag. *Escherichia coli* BL21(DE3) pLysS cells were transformed
601 with the constructs and cultured at 28°C. Protein expression was induced by the addition of 0.3
602 mM IPTG for 3 h at 28°C. The MBP/His-tagged proteins were purified using Ni²⁺-NTA agarose
603 (Qiagen) according to the manufacturer's instructions.

604

605 **Nuclear extraction**

606 Five-week-old leaves were ground in liquid nitrogen and incubated in a nuclear extraction
607 buffer (20 mM PIPES-KOH, pH 7.0, 10 mM KCl, 1.5 mM MgCl₂, 0.3% Triton X-100, 5 mM
608 EDTA, 1 mM DTT, 1 M 2-methyl-2,4-pentandiol, 1 mM NaF, 1 mM Na₃VO₄, and protease
609 inhibitor cocktail) on ice for 10 min. The material was filtered through one layer of Miracloth
610 and spun at 1,000 g for 10 min at 4°C. After removing the supernatant, the pellet was
611 resuspended in a buffer (20 mM PIPES-KOH, pH 7.0, 10 mM MgCl₂, 1% Triton X-100, 1 mM
612 DTT, 0.5 M hexylene glycol (2-methyl-2,4-pentandiol), 1 mM NaF, and 1 mM Na₃VO₄),
613 incubated on ice for 10 min, and then centrifuged at 1,000 g for 10 min at 4°C. To extract
614 nuclear proteins, isolated nuclei were resuspended in an extraction buffer (20 mM HEPES, pH
615 8.0, 300 mM NaCl, 1 mM MgCl₂, 0.2 mM EDTA, 10% glycerol, 1% Triton X-100, 0.1% NP-
616 40, 1 mM DTT, 1 mM NaF, 1 mM Na₃VO₄, and protease inhibitor cocktail), incubated with
617 rotation for 30 min at 4°C, and then centrifuged at 15,000 g for 20 min at 4°C. The supernatant
618 was collected, and the protein concentration was determined before use. For phosphatase
619 treatment, phosphatase inhibitors (NaF and Na₃VO₄) were excluded from the extraction buffer,
620 and extracted nuclear proteins were treated with lambda protein phosphatase (NEB) according
621 to the manufacturer's instruction.

622

623 **Electrophoretic mobility shift assay (EMSA)**

624 EMSA was performed using the LightShift Chemiluminescent EMSA kit (Thermo Scientific).
625 Biotin-labeled oligonucleotides were synthesized by Macrogen (Korea). Purified proteins or
626 nuclear extracts were incubated in 20 fM biotin-labeled oligonucleotide probes in 15 μ l of a
627 binding buffer (10 mM Tris-HCl, pH 7.5, 40 mM KCl, 3 mM MgCl₂, 1 mM EDTA, 10%
628 glycerol, 1 mM DTT, and 3 μ g poly(dI-dC)) for 30 min at room temperature (purified proteins)
629 or at 4°C (nuclear extracts). The samples were resolved on 5% polyacrylamide (75:1
630 acrylamide:bis-acrylamide) gels. In the competition assay, purified ORA59 proteins were
631 incubated with the indicated excess amounts of oligonucleotide competitors for 15 min before
632 the addition of biotin-labeled probes.

633

634 **Immunoblotting and immunoprecipitation**

635 For immunoblotting, proteins were separated on 10-12% SDS polyacrylamide gels by SDS-gel
636 electrophoresis and electro transferred onto nitrocellulose membranes. Membranes were
637 incubated with anti-GFP (sc-9996, Santa Cruz Biotechnology), anti-Actin (ab197345, Abcam),
638 anti-Histone H3 (ab1791, Abcam), and anti phospho Ser/Thr (ab17464, Abcam) antibodies. For
639 immunoprecipitation, nuclear pellets were lysed in hypotonic buffer (20 mM HEPES, pH 7.9,
640 20 mM KCl, 1.5 mM MgCl₂, and 25% glycerol) and high-salt buffer (20 mM HEPES, pH 7.9,
641 800 mM KCl, 1.5 mM MgCl₂, 25% glycerol, and 1% NP-40) supplemented with protease
642 inhibitor cocktail and incubated with rotation at 4°C. Lysates were cleared by centrifugation
643 and incubated with an anti-GFP antibody for 2 h at 4 °C. After an additional 2 h incubation
644 with Protein G Agarose (20399, Thermo Scientific), beads were washed with wash buffer (20
645 mM Tris-HCl, pH 7.9, 150 mM KCl, 20% glycerol, 0.1mM EDTA, and 0.1% NP-40) and
646 bound proteins were eluted with 2x sample buffer (100 mM Tris-HCl, pH 6.5, 20% glycerol,
647 4% SDS, 200 mM DTT, and 3 mM bromophenol blue). Immunoblot bands were visualized

648 using the enhanced chemiluminescence system (Amersham Biosciences).

649

650 **Gene expression analysis**

651 Total RNAs were extracted using TRIzol reagent and reverse-transcribed into cDNAs using the
652 PrimeScript RT reagent kit (TaKaRa). RT-qPCR was performed using KAPA SYBR FAST
653 qPCR master mix (Kapa Biosystems) with gene-specific primers (Supplementary Table 6) on
654 a LightCycler 480 system (Roche) according to the manufacturer's protocol. For transcript
655 normalization, *Actin1* was used as a reference gene. Data were analyzed using
656 LC480Conversion and LinRegPCR software (Heart Failure Research Center).

657

658 **RNA sequencing data analysis**

659 Total RNAs were extracted from leaves using RNeasy Plant Mini kit (Qiagen). The amount of
660 RNAs was measured using Nanodrop (Thermo Scientific) and the quality was assessed using
661 Bioanalyzer (Agilent Technologies) with an RNA Integrity Number (RIN) value ≥ 8 . The
662 RNA-seq libraries were prepared using the TruSeq RNA preparation kit V2 kit following the
663 manufacturer's instructions. The 150-bp paired-end sequencing reads were generated on the
664 Illumina NextSeq 550 System instrument platform. The low-quality base (base quality score $<$
665 20) in the last position of the reads was trimmed and high-quality sequencing reads were
666 subsequently aligned onto the *A. thaliana* reference genome (TAIR10) using HISAT2 (Kim et
667 al., 2019). The raw number of reads mapped onto each transcript was quantified using StringTie
668 (Pertea et al., 2015) and the counts per transcript were normalized based on the library size by
669 DESeq2 (Love et al., 2014). The batch effect among samples was estimated by PCA and
670 corrected by limma (Ritchie et al., 2015). Statistically significant DEGs were tested based on
671 a negative binomial distribution using a generalized linear model. Enriched GO terms for DEGs
672 were determined using the statistical overrepresentation test in PANTHER

673 (<http://geneontology.org>). Gene lists were compared to all *Arabidopsis* genes in PANTHER
674 using the GO BP dataset and binomial test with FDR correction.

675

676 **Chromatin immunoprecipitation (ChIP)**

677 ChIP assays were performed as described previously (Lee et al., 2017b). Five-week-old
678 *35S:ORA59-GFP* leaf tissues were fixed with 1% formaldehyde under vacuum, washed, dried,
679 and ground to a fine powder in liquid nitrogen. The powder was suspended in M1 buffer (10
680 mM sodium phosphate, pH 7.0, 0.1 M NaCl, 1 M 2-methyl 2,4 pentanediol, 10 mM β -
681 mercaptoethanol, and protease inhibitor cocktail). Nuclei were isolated from the filtrate by
682 centrifugation at 1000 g for 20 min at 4°C and washed with M2 buffer (10 mM sodium
683 phosphate, pH 7.0, 0.1 M NaCl, 1 M 2-methyl 2,4 pentanediol, 10 mM β -mercaptoethanol, 10
684 mM MgCl₂, 0.5% Triton X-100, and protease inhibitor cocktail) and M3 buffer (10 mM sodium
685 phosphate, pH 7.0, 0.1 M NaCl, 10 mM β -mercaptoethanol, and protease inhibitor cocktail).
686 The crude nuclear pellet was resuspended in sonication buffer (10 mM sodium phosphate, pH
687 7.0, 0.1M NaCl, 0.5% Sarkosyl, and 10 mM EDTA) and sonicated to obtain DNA fragments.
688 The fragmented chromatin was transferred to IP buffer (50 mM HEPES, pH 7.5, 150 mM NaCl,
689 5 mM MgCl₂, 10 μ M ZnSO₄, 1% Triton-X 100, and 0.05% SDS). The pre-cleared chromatin
690 was incubated with IgG or GFP antibody (A11122, Thermo Scientific) for 2 h at 4°C. After an
691 additional overnight incubation with Protein A Sepharose (20333, Thermo Scientific), beads
692 were incubated in elution buffer (0.1M glycine, pH 2.8, 0.5 M NaCl, 0.05% Triton X-100).
693 Primers for qPCR are listed in Supplementary Table 6.

694

695 **Virus-induced gene silencing (VIGS)**

696 VIGS was performed as previously described (Burch-Smith et al., 2006). Coding regions of
697 target genes were amplified and cloned into the pTRV2 vector. The constructs were

698 transformed into *Agrobacterium tumefaciens* strain GV3101, which was cultured in LB media
699 containing 10 mM MES-KOH (pH 5.7), 200 μ M acetosyringone, 50 mg l⁻¹ gentamycin, and 50
700 mg l⁻¹ kanamycin overnight at 28°C. *A. tumefaciens* cells were harvested, adjusted to an OD600
701 of 1.5 in infiltration media (10 mM MES-KOH, pH 5.7, 10 mM MgCl₂, and 200 μ M
702 acetosyringone), and infiltrated into leaves of *Arabidopsis* seedlings at 15-17 days after
703 germination. After 19-21 days, VIGS plants were treated with pathogens and silencing effects
704 were verified in the systemic leaves by gene-specific primers (Supplementary Table 6).

705

706 **Statistical analysis**

707 Statistical analyses were performed using GraphPad Prism (v. 8.0). Significant differences
708 between experimental groups were analyzed by one-way ANOVA with Tukey's HSD test or
709 unpaired Student's *t* test for multiple comparisons or single comparisons, respectively. Detailed
710 information about statistical analysis is described in the figure legends. Statistical significance
711 was set at $P < 0.05$. All experiments were repeated 3-5 times with similar results.

712 **References**

713

714 Adio, A.M., Casteel, C.L., De Vos, M., Kim, J.H., Joshi, V., Li, B., Juéry, C., Daron, J.,

715 Kliebenstein, D.J., and Jander, G. (2011). Biosynthesis and defensive function of Nδ-

716 acetyloronithine, a jasmonate-induced Arabidopsis metabolite. *Plant Cell* 23, 3303-3318.

717 Ahn, H.K., Kang, Y.W., Lim, H.M., Hwang, I., and Pai, H.S. (2015). Physiological Functions

718 of the COPI Complex in Higher Plants. *Mol. Cells* 38, 866-875.

719 Alonso, J.M., Hirayama, T., Roman, G., Nourizadeh, S., and Ecker, J.R. (1999). EIN2, a

720 bifunctional transducer of ethylene and stress responses in Arabidopsis. *Science* 284, 2148-

721 2152.

722 Alonso, J.M., Stepanova, A.N., Solano, R., Wisman, E., Ferrari, S., Ausubel, F.M., and Ecker,

723 J.R. (2003). Five components of the ethylene-response pathway identified in a screen for

724 weak ethylene-insensitive mutants in Arabidopsis. *Proc. Natl. Acad. Sci. USA* 100, 2992-

725 2997.

726 An, F., Zhang, X., Zhu, Z., Ji, Y., He, W., Jiang, Z., Li, M., and Guo, H. (2012). Coordinated

727 regulation of apical hook development by gibberellins and ethylene in etiolated Arabidopsis

728 seedlings. *Cell Res.* 22, 915-927.

729 Bäckström, S., Elfving, N., Nilsson, R., Wingsle, G., and Björklund, S. (2007). Purification of

730 a plant mediator from Arabidopsis thaliana identifies PFT1 as the Med25 subunit. *Mol. Cell*

731 26, 717-729.

732 Bednarek, P. (2012). Sulfur-containing secondary metabolites from Arabidopsis thaliana and

733 other Brassicaceae with function in plant immunity. *Chembiochem* 13, 1846-1859.

734 Berrocal-Lobo, M., Molina, A., and Solano, R. (2002). Constitutive expression of

735 ETHYLENE-RESPONSE-FACTOR1 in Arabidopsis confers resistance to several

736 necrotrophic fungi. *Plant J.* 29, 23-32.

- 737 Birkenbihl, R.P., Diezel, C., and Somssich, I.E. (2012). Arabidopsis WRKY33 is a key
738 transcriptional regulator of hormonal and metabolic responses toward *Botrytis cinerea*
739 infection. *Plant Physiol.* *159*, 266-285.
- 740 Bostock, R.M. (2005). Signal crosstalk and induced resistance: straddling the line between cost
741 and benefit. *Annu. Rev. Phytopathol.* *43*, 545-580.
- 742 Brodersen, P., Petersen, M., Bjørn Nielsen, H., Zhu, S., Newman, M.A., Shokat, K.M., Rietz,
743 S., Parker, J., and Mundy, J. (2006). Arabidopsis MAP kinase 4 regulates salicylic acid- and
744 jasmonic acid/ethylene-dependent responses via EDS1 and PAD4. *Plant J.* *47*, 532-546.
- 745 Broekaert, W.F., Terras, F.R., Cammue, B.P., and Vanderleyden, J. (1990). An automated
746 quantitative assay for fungal growth inhibition. *FEMS Microbiol. Lett.* *69*, 55-59.
- 747 Broekgaarden, C., Caarls, L., Vos, I.A., Pieterse, C.M., and Van Wees, S.C. (2015). Ethylene:
748 traffic controller on hormonal crossroads to defense. *Plant Physiol.* *169*, 2371-2379.
- 749 Brown, R.L., Kazan, K., McGrath, K.C., Maclean, D.J., and Manners, J.M. (2003). A role for
750 the GCC-box in jasmonate-mediated activation of the PDF1.2 gene of Arabidopsis. *Plant*
751 *Physiol.* *132*, 1020-1032.
- 752 Burch-Smith, T.M., Schiff, M., Liu, Y., and Dinesh-Kumar, S.P. (2006). Efficient virus-
753 induced gene silencing in Arabidopsis. *Plant Physiol.* *142*, 21-27.
- 754 Çevik, V., Kidd, B.N., Zhang, P., Hill, C., Kiddle, S., Denby, K.J., Holub, E.B., Cahill, D.M.,
755 Manners, J.M., and Schenk, P.M. (2012). MEDIATOR25 acts as an integrative hub for the
756 regulation of jasmonate-responsive gene expression in Arabidopsis. *Plant Physiol.* *160*, 541-
757 555.
- 758 Chang, K.N., Zhong, S., Weirauch, M.T., Hon, G., Pelizzola, M., Li, H., Huang, S.-s.C.,
759 Schmitz, R.J., Urich, M.A., and Kuo, D. (2013). Temporal transcriptional response to
760 ethylene gas drives growth hormone cross-regulation in Arabidopsis. *Elife* *2*, e00675.
- 761 Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., and Ecker, J.R. (1997).

- 762 Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein
763 ETHYLENE-INSENSITIVE3 and related proteins. *Cell* 89, 1133-1144.
- 764 Cheng, M.-C., Liao, P.-M., Kuo, W.-W., and Lin, T.-P. (2013). The Arabidopsis ETHYLENE
765 RESPONSE FACTOR1 regulates abiotic stress-responsive gene expression by binding to
766 different cis-acting elements in response to different stress signals. *Plant Physiol.* 162, 1566-
767 1582.
- 768 Cheng, Z., Sun, L., Qi, T., Zhang, B., Peng, W., Liu, Y., and Xie, D. (2011). The bHLH
769 transcription factor MYC3 interacts with the Jasmonate ZIM-domain proteins to mediate
770 jasmonate response in Arabidopsis. *Mol. Plant* 4, 279-288.
- 771 Cheong, Y.H., Moon, B.C., Kim, J.K., Kim, C.Y., Kim, M.C., Kim, I.H., Park, C.Y., Kim, J.C.,
772 Park, B.O., and Koo, S.C. (2003). BWMK1, a rice mitogen-activated protein kinase, locates
773 in the nucleus and mediates pathogenesis-related gene expression by activation of a
774 transcription factor. *Plant Physiol.* 132, 1961-1972.
- 775 Chini, A., Fonseca, S., Fernández, G., Adie, B., Chico, J.M., Lorenzo, O., García-Casado, G.,
776 López-Vidriero, I., Lozano, F.M., Ponce, M.R., *et al.* (2007). The JAZ family of repressors
777 is the missing link in jasmonate signalling. *Nature* 448, 666-671.
- 778 Cho, Y.H., and Yoo, S.D. (2011). Signaling role of fructose mediated by FINS1/FBP in
779 Arabidopsis thaliana. *PLoS Genet.* 7, e1001263.
- 780 Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-
781 mediated transformation of Arabidopsis thaliana. *Plant J.* 16, 735-743.
- 782 De Boer, K., Tilleman, S., Pauwels, L., Vanden Bossche, R., De Sutter, V., Vanderhaeghen,
783 R., Hilson, P., Hamill, J.D., and Goossens, A. (2011). APETALA2/ETHYLENE
784 RESPONSE FACTOR and basic helix–loop–helix tobacco transcription factors
785 cooperatively mediate jasmonate-elicited nicotine biosynthesis. *Plant J.* 66, 1053-1065.
- 786 De Vos, M., Van Oosten, V.R., Van Poecke, R.M., Van Pelt, J.A., Pozo, M.J., Mueller, M.J.,

- 787 Buchala, A.J., Métraux, J.-P., Van Loon, L.C., and Dicke, M. (2005). Signal signature and
788 transcriptome changes of Arabidopsis during pathogen and insect attack. *Mol. Plant*
789 *Microbe Interact.* *18*, 923-937.
- 790 Dong, X. (1998). SA, JA, ethylene, and disease resistance in plants. *Curr. Opin. Plant Biol.* *1*,
791 316-323.
- 792 Eichmann, R., and Schäfer, P. (2012). The endoplasmic reticulum in plant immunity and cell
793 death. *Front. Plant Sci.* *3*, 200.
- 794 Fernández-Calvo, P., Chini, A., Fernández-Barbero, G., Chico, J.M., Gimenez-Ibanez, S.,
795 Geerinck, J., Eeckhout, D., Schweizer, F., Godoy, M., Franco-Zorrilla, J.M., *et al.* (2011).
796 The Arabidopsis bHLH transcription factors MYC3 and MYC4 are targets of JAZ
797 repressors and act additively with MYC2 in the activation of jasmonate responses. *Plant*
798 *Cell* *23*, 701-715.
- 799 Feys, B.J., and Parker, J.E. (2000). Interplay of signaling pathways in plant disease resistance.
800 *Trends Genet.* *16*, 449-455.
- 801 Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic
802 pathogens. *Annu. Rev. Phytopathol.* *43*, 205-227.
- 803 Gosti, F., Bertauche, N., Vartanian, N., and Giraudat, J. (1995). Abscisic acid-dependent and -
804 independent regulation of gene expression by progressive drought in *Arabidopsis thaliana*.
805 *Mol. Gen. Genet.* *246*, 10-18.
- 806 Gu, Y.-Q., Yang, C., Thara, V.K., Zhou, J., and Martin, G.B. (2000). Pti4 is induced by
807 ethylene and salicylic acid, and its product is phosphorylated by the Pto kinase. *Plant Cell*
808 *12*, 771-785.
- 809 Guo, H., and Ecker, J.R. (2003). Plant responses to ethylene gas are mediated by
810 SCFEBF1/EBF2-dependent proteolysis of EIN3 transcription factor. *Cell* *115*, 667-677.
- 811 Hao, D., Ohme-Takagi, M., and Sarai, A. (1998). Unique mode of GCC box recognition by the

- 812 DNA-binding domain of ethylene-responsive element-binding factor (ERF domain) in plant.
813 *J. Biol. Chem.* *273*, 26857-26861.
- 814 He, X., Jiang, J., Wang, C.Q., and Dehesh, K. (2017). ORA59 and EIN3 interaction couples
815 jasmonate-ethylene synergistic action to antagonistic salicylic acid regulation of PDF
816 expression. *J. Integr. Plant Biol.* *59*, 275-287.
- 817 Hickman, R., Van Verk, M.C., Van Dijken, A.J., Mendes, M.P., Vroegop-Vos, I.A., Caarls, L.,
818 Steenbergen, M., Van der Nagel, I., Wesselink, G.J., and Jironkin, A. (2017). Architecture
819 and dynamics of the jasmonic acid gene regulatory network. *Plant Cell* *29*, 2086-2105.
- 820 Huang, P., Dong, Z., Guo, P., Zhang, X., Qiu, Y., Li, B., Wang, Y., and Guo, H. (2020).
821 Salicylic acid suppresses apical hook formation via NPR1-mediated repression of EIN3 and
822 EIL1 in Arabidopsis. *Plant Cell* *32*, 612-629.
- 823 Huang, P.-Y., Catinot, J., and Zimmerli, L. (2016). Ethylene response factors in Arabidopsis
824 immunity. *J. Exp. Bot.* *67*, 1231-1241.
- 825 Itzhaki, H., Maxson, J.M., and Woodson, W.R. (1994). An ethylene-responsive enhancer
826 element is involved in the senescence-related expression of the carnation glutathione-S-
827 transferase (GST1) gene. *Proc. Natl. Acad. Sci. USA* *91*, 8925-8929.
- 828 Joo, S., and Kim, W.T. (2007). A gaseous plant hormone ethylene: The signaling pathway. *J.*
829 *Plant Biol.* *50*, 109-116.
- 830 Ju, C., Yoon, G.M., Shemansky, J.M., Lin, D.Y., Ying, Z.I., Chang, J., Garrett, W.M.,
831 Kessenbrock, M., Groth, G., Tucker, M.L., *et al.* (2012). CTR1 phosphorylates the central
832 regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus
833 in Arabidopsis. *Proc. Natl. Acad. Sci. USA* *109*, 19486-19491.
- 834 Kagaya, Y., Ohmiya, K., and Hattori, T. (1999). RAV1, a novel DNA-binding protein, binds
835 to bipartite recognition sequence through two distinct DNA-binding domains uniquely
836 found in higher plants. *Nucleic Acids Res.* *27*, 470-478.

- 837 Katsir, L., Schilmiller, A.L., Staswick, P.E., He, S.Y., and Howe, G.A. (2008). COI1 is a
838 critical component of a receptor for jasmonate and the bacterial virulence factor coronatine.
839 Proc. Natl. Acad. Sci. USA *105*, 7100-7105.
- 840 Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A., and Ecker, J.R. (1993). CTR1, a
841 negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of
842 the raf family of protein kinases. Cell *72*, 427-441.
- 843 Kim, D., Paggi, J.M., Park, C., Bennett, C., and Salzberg, S.L. (2019). Graph-based genome
844 alignment and genotyping with HISAT2 and HISAT-genotype. Nat. Biotechnol. *37*, 907-
845 915.
- 846 Kim, H.G., Kwon, S.J., Jang, Y.J., Chung, J.H., Nam, M.H., and Park, O.K. (2014). GDLSL
847 lipase 1 regulates ethylene signaling and ethylene-associated systemic immunity in
848 Arabidopsis. FEBS lett. *588*, 1652-1658.
- 849 Kim, H.G., Kwon, S.J., Jang, Y.J., Nam, M.H., Chung, J.H., Na, Y.-C., Guo, H., and Park, O.K.
850 (2013). GDLSL LIPASE1 modulates plant immunity through feedback regulation of ethylene
851 signaling. Plant Physiol. *163*, 1776-1791.
- 852 Kim, N.Y., Jang, Y.J., and Park, O.K. (2018). AP2/ERF family transcription factors ORA59
853 and RAP2.3 interact in the nucleus and function together in ethylene responses. Front. Plant
854 Sci. *9*, 1675.
- 855 Konishi, M., and Yanagisawa, S. (2008). Ethylene signaling in Arabidopsis involves feedback
856 regulation via the elaborate control of EBF2 expression by EIN3. Plant J. *55*, 821-831.
- 857 Koornneef, A., and Pieterse, C.M. (2008). Cross talk in defense signaling. Plant Physiol. *146*,
858 839-844.
- 859 Kunkel, B.N., and Brooks, D.M. (2002). Cross talk between signaling pathways in pathogen
860 defense. Curr. Opin. Plant Biol. *5*, 325-331.
- 861 Kwon, S.J., Jin, H.C., Lee, S., Nam, M.H., Chung, J.H., Kwon, S.I., Ryu, C.M., and Park, O.K.

- 862 (2009). GDSL lipase-like 1 regulates systemic resistance associated with ethylene signaling
863 in Arabidopsis. *Plant J.* 58, 235-245.
- 864 Laluk, K., and Mengiste, T. (2011). The Arabidopsis extracellular UNUSUAL SERINE
865 PROTEASE INHIBITOR functions in resistance to necrotrophic fungi and insect herbivory.
866 *Plant J.* 68, 480-494.
- 867 Lee, D.S., Kim, Y.C., Kwon, S.J., Ryu, C.M., and Park, O.K. (2017a). The Arabidopsis
868 Cysteine-Rich Receptor-Like Kinase CRK36 Regulates Immunity through Interaction with
869 the Cytoplasmic Kinase BIK1. *Front. Plant Sci.* 8, 1856.
- 870 Lee, M.H., Jeon, H.S., Kim, H.G., and Park, O.K. (2017b). An Arabidopsis NAC transcription
871 factor NAC4 promotes pathogen-induced cell death under negative regulation by
872 microRNA164. *New Phytol.* 214, 343-360.
- 873 Li, Z., Peng, J., Wen, X., and Guo, H. (2013). Ethylene-insensitive3 is a senescence-associated
874 gene that accelerates age-dependent leaf senescence by directly repressing miR164
875 transcription in Arabidopsis. *Plant Cell* 25, 3311-3328.
- 876 Licausi, F., Ohme-Takagi, M., and Perata, P. (2013). APETALA 2/Ethylene Responsive Factor
877 (AP 2/ERF) transcription factors: Mediators of stress responses and developmental
878 programs. *New Phytol.* 199, 639-649.
- 879 Lorenzo, O., Piqueras, R., Sánchez-Serrano, J.J., and Solano, R. (2003). ETHYLENE
880 RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant
881 defense. *Plant Cell* 15, 165-178.
- 882 Lou, Y.R., Bor, M., Yan, J., Preuss, A.S., and Jander, G. (2016). Arabidopsis NATA1
883 Acetylates Putrescine and Decreases Defense-Related Hydrogen Peroxide Accumulation.
884 *Plant Physiol.* 171, 1443-1455.
- 885 Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and
886 dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 550.

- 887 Meng, X., Xu, J., He, Y., Yang, K.-Y., Mordorski, B., Liu, Y., and Zhang, S. (2013).
888 Phosphorylation of an ERF transcription factor by Arabidopsis MPK3/MPK6 regulates
889 plant defense gene induction and fungal resistance. *Plant Cell* 25, 1126-1142.
- 890 Montgomery, J., Goldman, S., Deikman, J., Margossian, L., and Fischer, R.L. (1993).
891 Identification of an ethylene-responsive region in the promoter of a fruit ripening gene. *Proc.*
892 *Natl. Acad. Sci. USA* 90, 5939-5943.
- 893 Morikawa-Ichinose, T., Miura, D., Zhang, L., Kim, S.J., and Maruyama-Nakashita, A. (2020).
894 Involvement of BGLU30 in Glucosinolate Catabolism in the Arabidopsis Leaf under Dark
895 Conditions. *Plant Cell Physiol.* 61, 1095-1106.
- 896 Niu, Y., Figueroa, P., and Browse, J. (2011). Characterization of JAZ-interacting bHLH
897 transcription factors that regulate jasmonate responses in Arabidopsis. *J. Exp. Bot.* 62, 2143-
898 2154.
- 899 Oh, I.S., Park, A.R., Bae, M.S., Kwon, S.J., Kim, Y.S., Lee, J.E., Kang, N.Y., Lee, S., Cheong,
900 H., and Park, O.K. (2005). Secretome analysis reveals an Arabidopsis lipase involved in
901 defense against *Alternaria brassicicola*. *Plant Cell* 17, 2832-2847.
- 902 Ohme-Takagi, M., and Shinshi, H. (1995). Ethylene-inducible DNA binding proteins that
903 interact with an ethylene-responsive element. *Plant Cell* 7, 173-182.
- 904 Ou, B., Yin, K.-Q., Liu, S.-N., Yang, Y., Gu, T., Hui, J.M.W., Zhang, L., Miao, J., Kondou,
905 Y., and Matsui, M. (2011). A high-throughput screening system for Arabidopsis
906 transcription factors and its application to Med25-dependent transcriptional regulation. *Mol.*
907 *Plant* 4, 546-555.
- 908 Penninckx, I.A., Thomma, B.P., Buchala, A., Métraux, J.-P., and Broekaert, W.F. (1998).
909 Concomitant activation of jasmonate and ethylene response pathways is required for
910 induction of a plant defensin gene in Arabidopsis. *Plant Cell* 10, 2103-2113.
- 911 Pertea, M., Pertea, G.M., Antonescu, C.M., Chang, T.C., Mendell, J.T., and Salzberg, S.L.

- 912 (2015). StringTie enables improved reconstruction of a transcriptome from RNA-seq reads.
913 *Nat. Biotechnol.* *33*, 290-295.
- 914 Pfalz, M., Mikkelsen, M.D., Bednarek, P., Olsen, C.E., Halkier, B.A., and Kroymann, J. (2011).
915 Metabolic engineering in *Nicotiana benthamiana* reveals key enzyme functions in
916 *Arabidopsis* indole glucosinolate modification. *Plant Cell* *23*, 716-729.
- 917 Phukan, U.J., Jeena, G.S., Tripathi, V., and Shukla, R.K. (2017). Regulation of
918 *Apetala2*/Ethylene response factors in plants. *Front. Plant Sci.* *8*, 150.
- 919 Pieterse, C.M., Leon-Reyes, A., Van der Ent, S., and Van Wees, S.C. (2009). Networking by
920 small-molecule hormones in plant immunity. *Nat. Chem. Biol.* *5*, 308-316.
- 921 Pieterse, C.M., Van der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S.C. (2012).
922 Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* *28*, 489-521.
- 923 Pogorelko, G.V., Mokryakova, M., Fursova, O.V., Abdeeva, I., Piruzian, E.S., and Bruskin,
924 S.A. (2014). Characterization of three *Arabidopsis thaliana* immunophilin genes involved
925 in the plant defense response against *Pseudomonas syringae*. *Gene* *538*, 12-22.
- 926 Potuschak, T., Lechner, E., Parmentier, Y., Yanagisawa, S., Grava, S., Koncz, C., and Genschik,
927 P. (2003). EIN3-dependent regulation of plant ethylene hormone signaling by two
928 *Arabidopsis* F box proteins: EBF1 and EBF2. *Cell* *115*, 679-689.
- 929 Pré, M., Atallah, M., Champion, A., De Vos, M., Pieterse, C.M., and Memelink, J. (2008a).
930 The AP2/ERF domain transcription factor *ORA59* integrates jasmonic acid and ethylene
931 signals in plant defense. *Plant Physiol.* *147*, 1347-1357.
- 932 Pré, M., Atallah, M., Champion, A., De Vos, M., Pieterse, C.M., and Memelink, J. (2008b).
933 The AP2/ERF domain transcription factor *ORA59* integrates jasmonic acid and ethylene
934 signals in plant defense. *Plant Physiol.* *147*, 1347-1357.
- 935 Qiao, H., Shen, Z., Huang, S.S., Schmitz, R.J., Urich, M.A., Briggs, S.P., and Ecker, J.R.
936 (2012). Processing and subcellular trafficking of ER-tethered EIN2 control response to

- 937 ethylene gas. *Science* 338, 390-393.
- 938 Reyes, F., Marchant, L., Norambuena, L., Nilo, R., Silva, H., and Orellana, A. (2006). AtUTr1,
939 a UDP-glucose/UDP-galactose transporter from *Arabidopsis thaliana*, is located in the
940 endoplasmic reticulum and up-regulated by the unfolded protein response. *J. Biol. Chem.*
941 281, 9145-9151.
- 942 Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W., and Smyth, G.K. (2015).
943 limma powers differential expression analyses for RNA-sequencing and microarray studies.
944 *Nucleic Acids Res.* 43, e47.
- 945 Roman, G., and Ecker, J.R. (1995). Genetic analysis of a seedling stress response to ethylene
946 in *Arabidopsis*. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 350, 75-81.
- 947 Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C., and
948 Manners, J.M. (2000). Coordinated plant defense responses in *Arabidopsis* revealed by
949 microarray analysis. *Proc. Natl. Acad. Sci. USA* 97, 11655-11660.
- 950 Sheard, L.B., Tan, X., Mao, H., Withers, J., Ben-Nissan, G., Hinds, T.R., Kobayashi, Y., Hsu,
951 F.F., Sharon, M., Browse, J., *et al.* (2010). Jasmonate perception by inositol-phosphate-
952 potentiated COI1-JAZ co-receptor. *Nature* 468, 400-405.
- 953 Solano, R., Stepanova, A., Chao, Q., and Ecker, J.R. (1998). Nuclear events in ethylene
954 signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and
955 ETHYLENE-RESPONSE-FACTOR1. *Genes Dev.* 12, 3703-3714.
- 956 Spoel, S.H., and Dong, X. (2008). Making sense of hormone crosstalk during plant immune
957 responses. *Cell Host Microbe* 3, 348-351.
- 958 Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S.Y.,
959 Howe, G.A., and Browse, J. (2007). JAZ repressor proteins are targets of the SCF(COI1)
960 complex during jasmonate signalling. *Nature* 448, 661-665.
- 961 Thomma, B.P., Eggermont, K., Penninckx, I.A., Mauch-Mani, B., Vogelsang, R., Cammue,

- 962 B.P., and Broekaert, W.F. (1998). Separate jasmonate-dependent and salicylate-dependent
963 defense-response pathways in Arabidopsis are essential for resistance to distinct microbial
964 pathogens. *Proc. Natl. Acad. Sci. USA* 95, 15107-15111.
- 965 Thomma, B.P., Eggermont, K., Tierens, K.F., and Broekaert, W.F. (1999). Requirement of
966 functional ethylene-insensitive 2 gene for efficient resistance of Arabidopsis to infection by
967 *Botrytis cinerea*. *Plant Physiol.* 121, 1093-1102.
- 968 Van der Does, D., Leon-Reyes, A., Koornneef, A., Van Verk, M.C., Rodenburg, N., Pauwels,
969 L., Goossens, A., Körbes, A.P., Memelink, J., and Ritsema, T. (2013). Salicylic acid
970 suppresses jasmonic acid signaling downstream of SCFCO11-JAZ by targeting GCC
971 promoter motifs via transcription factor ORA59. *Plant Cell* 25, 744-761.
- 972 Wang, P., Du, Y., Zhao, X., Miao, Y., and Song, C.-P. (2013). The MPK6-ERF6-ROS-
973 responsive cis-acting Element7/GCC box complex modulates oxidative gene transcription
974 and the oxidative response in Arabidopsis. *Plant Physiol.* 161, 1392-1408.
- 975 Welchen, E., Viola, I.L., Kim, H.J., Prendes, L.P., Comelli, R.N., Hong, J.C., and Gonzalez,
976 D.H. (2009). A segment containing a G-box and an ACGT motif confers differential
977 expression characteristics and responses to the Arabidopsis *Cytc-2* gene, encoding an
978 isoform of cytochrome c. *J. Exp. Bot.* 60, 829-845.
- 979 Wen, X., Zhang, C., Ji, Y., Zhao, Q., He, W., An, F., Jiang, L., and Guo, H. (2012). Activation
980 of ethylene signaling is mediated by nuclear translocation of the cleaved EIN2 carboxyl
981 terminus. *Cell Res.* 22, 1613-1616.
- 982 Yan, J., Zhang, C., Gu, M., Bai, Z., Zhang, W., Qi, T., Cheng, Z., Peng, W., Luo, H., Nan, F.,
983 *et al.* (2009). The Arabidopsis CORONATINE INSENSITIVE1 protein is a jasmonate
984 receptor. *Plant Cell* 21, 2220-2236.
- 985 Yang, Y., Ou, B., Zhang, J., Si, W., Gu, H., Qin, G., and Qu, L.J. (2014). The Arabidopsis
986 Mediator subunit MED16 regulates iron homeostasis by associating with EIN3/EIL1

- 987 through subunit MED25. *Plant J.* 77, 838-851.
- 988 Yang, Y.-X., J Ahammed, G., Wu, C., Fan, S.-y., and Zhou, Y.-H. (2015). Crosstalk among
989 jasmonate, salicylate and ethylene signaling pathways in plant disease and immune
990 responses. *Curr. Protein Pept. Sci.* 16, 450-461.
- 991 Yoo, S.D., Cho, Y.H., and Sheen, J. (2007). Arabidopsis mesophyll protoplasts: a versatile cell
992 system for transient gene expression analysis. *Nat. Protoc.* 2, 1565-1572.
- 993 Zander, M., Chen, S., Imkampe, J., Thurow, C., and Gatz, C. (2012). Repression of the
994 Arabidopsis thaliana jasmonic acid/ethylene-induced defense pathway by TGA-interacting
995 glutaredoxins depends on their C-terminal ALWL motif. *Mol. Plant* 5, 831-840.
- 996 Zander, M., Thurow, C., and Gatz, C. (2014). TGA transcription factors activate the salicylic
997 acid-suppressible branch of the ethylene-induced defense program by regulating ORA59
998 expression. *Plant Physiol.* 165, 1671-1683.
- 999 Zarei, A., Körbes, A.P., Younessi, P., Montiel, G., Champion, A., and Memelink, J. (2011).
1000 Two GCC boxes and AP2/ERF-domain transcription factor ORA59 in jasmonate/ethylene-
1001 mediated activation of the PDF1. 2 promoter in Arabidopsis. *Plant Mol. Biol.* 75, 321-331.
- 1002 Zhang, L., Kawaguchi, R., Morikawa-Ichinose, T., Allahham, A., Kim, S.J., and Maruyama-
1003 Nakashita, A. (2020). Sulfur Deficiency-Induced Glucosinolate Catabolism Attributed to
1004 Two β -Glucosidases, BGLU28 and BGLU30, is Required for Plant Growth Maintenance
1005 under Sulfur Deficiency. *Plant Cell Physiol.* 61, 803-813.
- 1006 Zhong, S., Zhao, M., Shi, T., Shi, H., An, F., Zhao, Q., and Guo, H. (2009). EIN3/EIL1
1007 cooperate with PIF1 to prevent photo-oxidation and to promote greening of Arabidopsis
1008 seedlings. *Proc. Natl. Acad. Sci. USA* 106, 21431-21436.
- 1009 Zhu, Z. (2014). Molecular basis for jasmonate and ethylene signal interactions in Arabidopsis.
1010 *J. Exp. Bot.* 65, 5743-5748.
- 1011 Zhu, Z., An, F., Feng, Y., Li, P., Xue, L., A, M., Jiang, Z., Kim, J.M., To, T.K., Li, W., *et al.*

- 1012 (2011). Derepression of ethylene-stabilized transcription factors (EIN3/EIL1) mediates
1013 jasmonate and ethylene signaling synergy in Arabidopsis. *Proc. Natl. Acad. Sci. USA* *108*,
1014 12539-12544.
- 1015 Zhu, Z., and Lee, B. (2015). Friends or foes: new insights in jasmonate and ethylene co-actions.
1016 *Plant Cell Physiol.* *56*, 414-420.

1017 **Acknowledgments**

1018 We are grateful to H. S. Pai (Yonsei University, Korea) for technical help with VIGS. This
1019 work was supported by a Korea University grant, a Next-Generation BioGreen 21 Program
1020 (SSAC, PJ013202) from the Rural Development Administration and a National Research
1021 Foundation of Korea (NRF) grant (2018R1A5A1023599, SRC) from the Korean government
1022 (MSIP). J.C.H. acknowledges support from a NRF grant (2020R1A6A1A03044344) from the
1023 Korean government (MSIP).

1024

1025 **Author contributions**

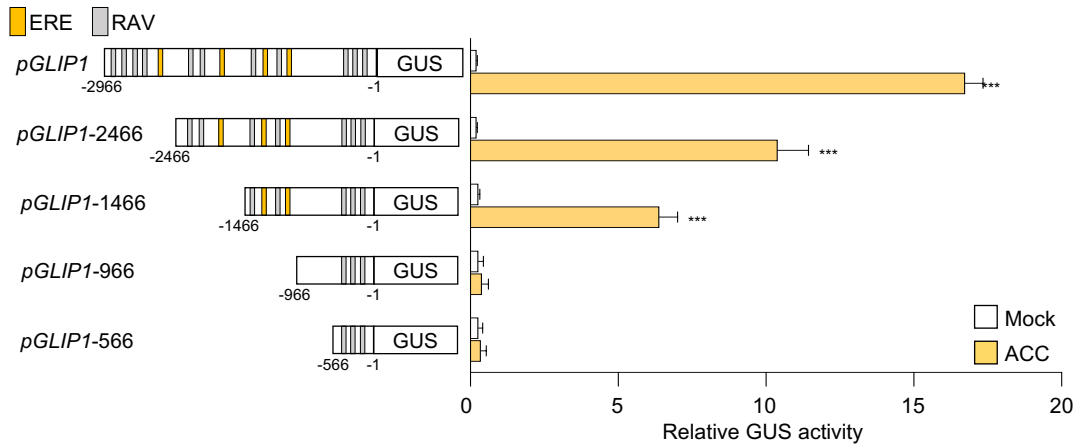
1026 O.K.P. conceived and directed the project. Y.N.Y. and O.K.P. designed the research. Y.N.Y.
1027 performed most of the experiments. Y.K. performed protein purification and EMSA analysis.
1028 H.K. conducted ChIP-qPCR analysis. D.S.L. and M.H.L. participated in EMSA and ChIP-
1029 qPCR analysis. S.J.K. generated transgenic and VIGS lines. K.M.C., Y.K., S.J.K., and J.C.
1030 conducted RNA-seq analysis. S.Y.K. and J.C.H. participated in Y1H screening experiments.
1031 Y.N.Y. and O.K.P. analyzed the data and wrote the manuscript. All authors contributed to the
1032 review and editing of the manuscript.

1033

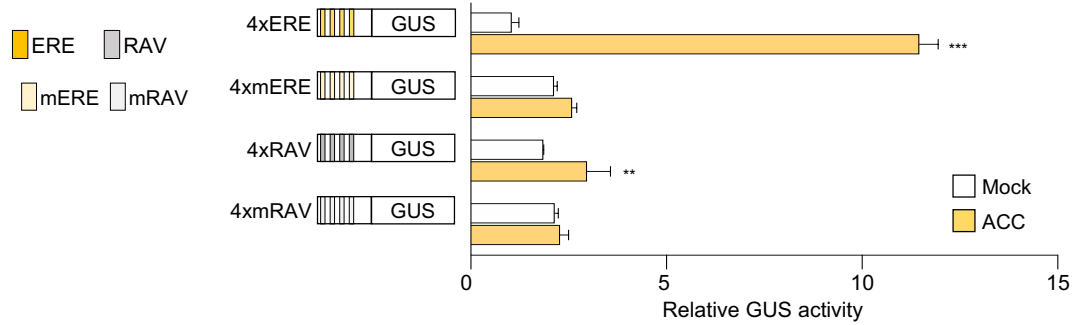
1034 **Competing interests**

1035 The authors declare no competing interests.

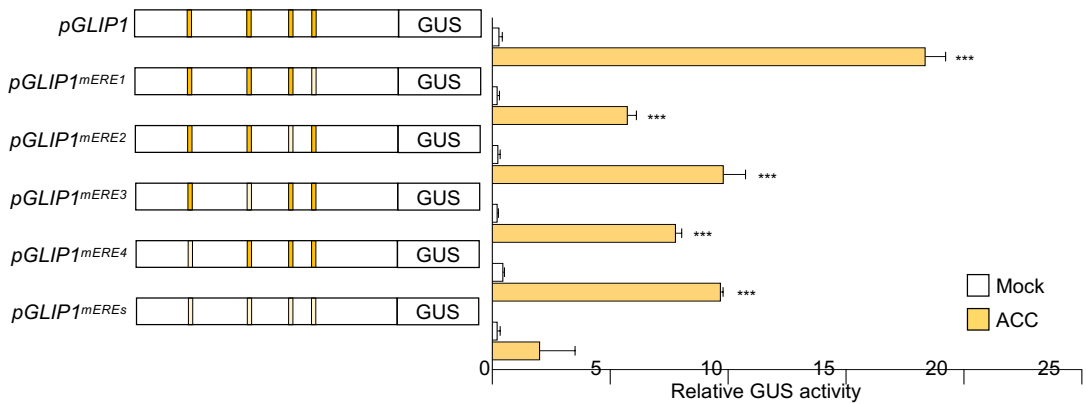
a



b



c



d

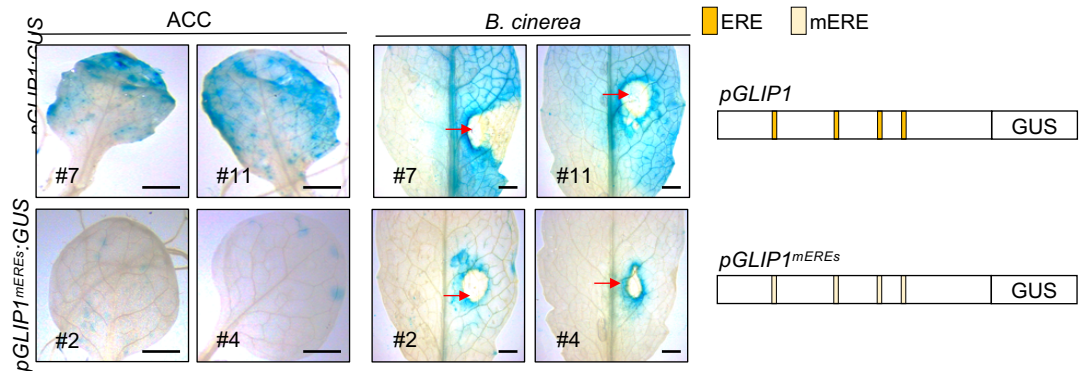


Figure 1. ERE is the essential regulatory element in the *GLIP1* promoter.

a, GUS reporter assays showing ACC-induced expression of the *GUS* reporter gene driven by the full-length and truncated *GLIP1* promoters. The left panel illustrates deletions of the *GLIP1* promoter. The ERE and RAV elements in the *GLIP1* promoter are boxed in yellow and gray, respectively. **b**, GUS reporter assays showing ACC-induced expression of the *GUS* reporter gene driven by synthetic promoters of 4xERE and 4xRAV, and their mutant versions 4xmERE and 4xmRAV. The left panel illustrates synthetic promoters. **c**, GUS reporter assays showing ACC-induced expression of the *GUS* reporter gene driven by the *GLIP1* promoters with individual or all ERE mutations. The left panel illustrates ERE mutations of the *GLIP1* promoter. **d**, GUS staining of ACC- and *B. cinerea*-treated leaves of transgenic plants expressing the *GUS* reporter gene driven by native or ERE-mutated *GLIP1* promoters. Six-week-old *pGLIP1:GUS* and *pGLIP1^{mERE}:GUS* plants were treated with ACC (1 mM) for 6 h or with 5 μ l droplets of *B. cinerea* spore suspensions (5×10^5 spores ml^{-1}) for 2 days. Representative images are provided, and infection sites are indicated by red arrows. Bars, 1 mm. In **a-c**, transfected protoplasts were treated with mock (water) and ACC (200 μ M) for 6 h. Values represent means \pm SD ($n = 3$ biological replicates). Asterisks indicate significant differences from mock treatment as determined by one-way ANOVA with Tukey test (** $P < 0.01$; *** $P < 0.001$).

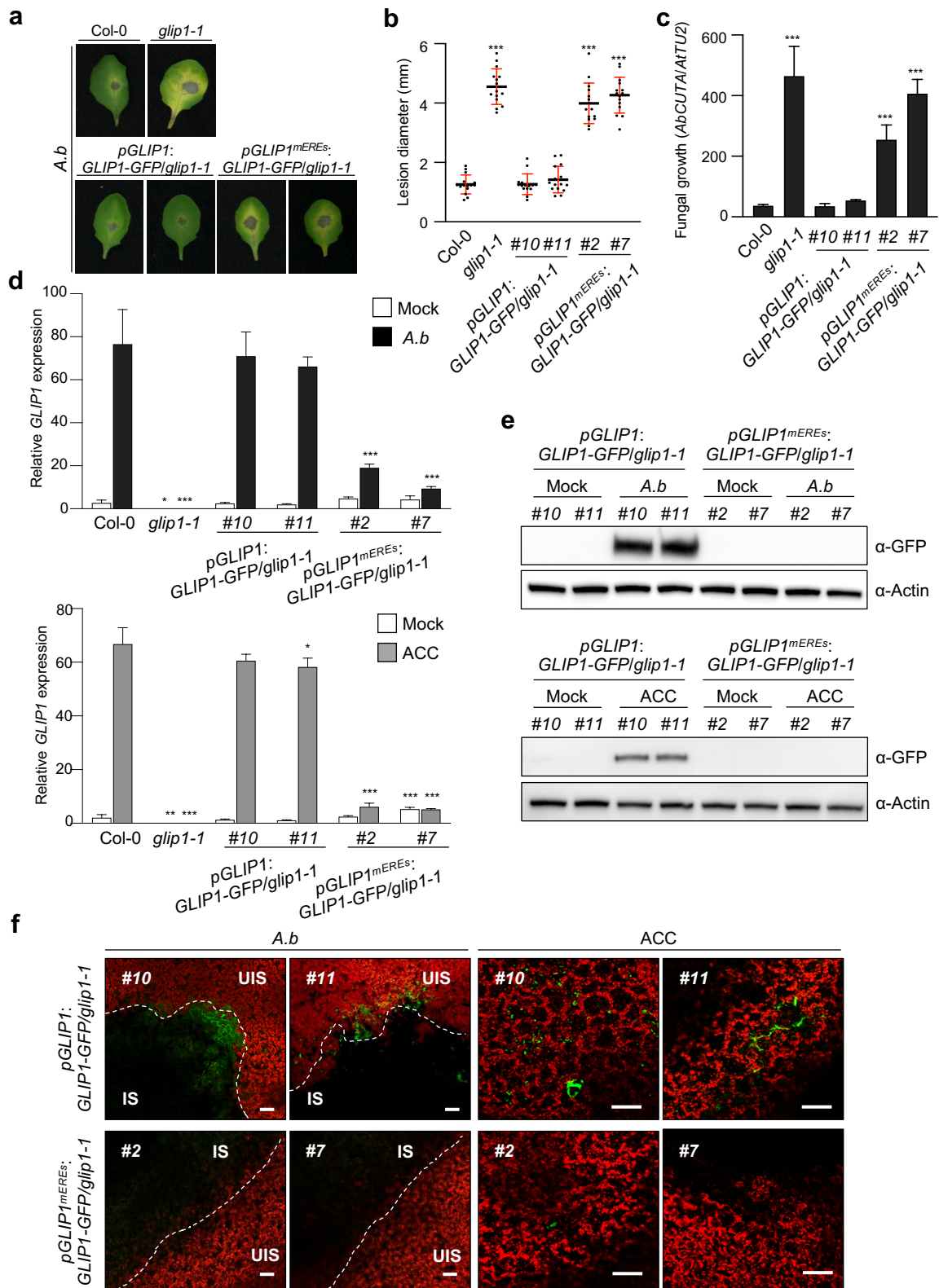


Figure 2. ERE is required for *GLIP1* expression during the immune response.

a,b, Disease symptoms (**a**) and lesion diameters (**b**) in leaves inoculated with *A. brassicicola*. Values represent means \pm SD ($n = 15$ infected leaves). **c**, Measurement of *A. brassicicola* growth in infected leaves. The abundance of *A. brassicicola cutinase A* (*AbCUTA*) gene relative to *Arabidopsis tubulin 2* (*AtTU2*) was analyzed by qPCR. Values represent means \pm SD ($n = 6$ infected leaves). **d**, Analysis of *GLIP1* expression in *A. brassicicola*- and ACC-treated plants. Values represent means \pm SD ($n = 3$ biological replicates). **e**, Immunoblot analysis of *GLIP1*-GFP expression in *A. brassicicola*- and ACC-treated plants. Protein extracts were subjected to immunoblotting with anti-GFP and anti-Actin antibodies. Actin levels served as a control. **f**, Confocal images of *GLIP1*-GFP expression in *A. brassicicola*- and ACC-treated plants. Bars, 100 μ m. Two independent transgenic lines were used in all experiments. Six-week-old plants were treated with ACC (1 mM) for 6 h (**d-f**) or with 5 μ l droplets of *B. cinerea* spore suspensions (5×10^5 spores ml⁻¹) for 1 (**d**) and 2 (**a,b,c,e,f**) days. In **b-d**, asterisks indicate significant differences from the respective Col-0 as determined by one-way ANOVA with Tukey test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). IS, infected site; UIS, uninfected site; *A.b*, *A. brassicicola*.

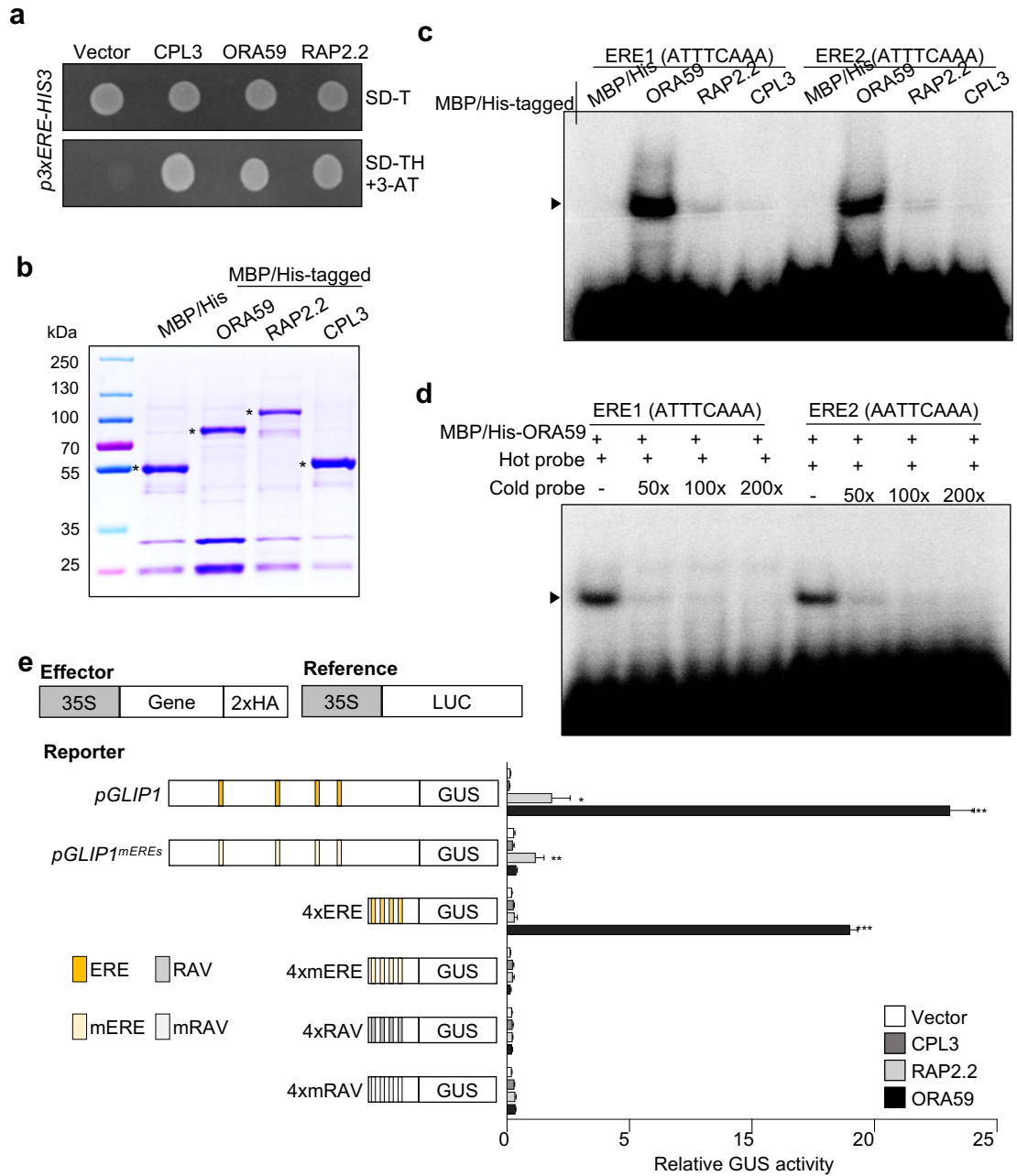


Figure 3. ORA59 is the specific ERE-binding transcription factor.

a, Isolation of ERE-binding transcription factors by Y1H screening. Yeast cells harboring the 3xERE-*HIS3* reporter gene were transformed with effector constructs *CPL3*, *ORA59*, and *RAP2.2*. Transcription factor binding to the ERE was tested on a selective medium lacking tryptophan and histidine, and supplemented with 0.2 mM 3-amino-1,2,4-triazole (SD-TH + 3-AT). **b**, Coomassie blue staining of purified recombinant *ORA59*, *RAP2.2*, and *CPL3* fused with N-terminal MBP and His tags. Asterisks indicate the corresponding purified proteins. **c**, DNA binding of *ORA59*, *RAP2.2*, and *CPL3* to the two ERE sequences ATTTCAA (ERE1) and AATTCAA (ERE2). Recombinant proteins were incubated with biotin-labeled ERE oligonucleotide probes in EMSA. **d**, Competition assays. *ORA59* binding to the ERE1/2 was competed with increasing amounts (50x, 100x, 200x) of unlabeled oligonucleotide competitors. **e**, Transactivation analysis showing the *ORA59*-mediated *GUS* reporter gene induction driven by the *GLIP1* and 4xERE synthetic promoters. The left panel illustrates reference, effector, and reporter constructs. Reporter DNAs, either alone or together with effector DNAs, were transfected into protoplasts, and *GUS* activity was measured. Luciferase (LUC) expressed under control of the CaMV 35S promoter was used as an internal control (reference). Values represent means \pm SD ($n = 3$ biological replicates). Asterisks indicate significant differences from vector control as determined by one-way ANOVA with Tukey test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). MBP, maltose-binding protein; 35S, CaMV 35S; 2xHA, two copies of the hemagglutinin (HA) tag sequence.

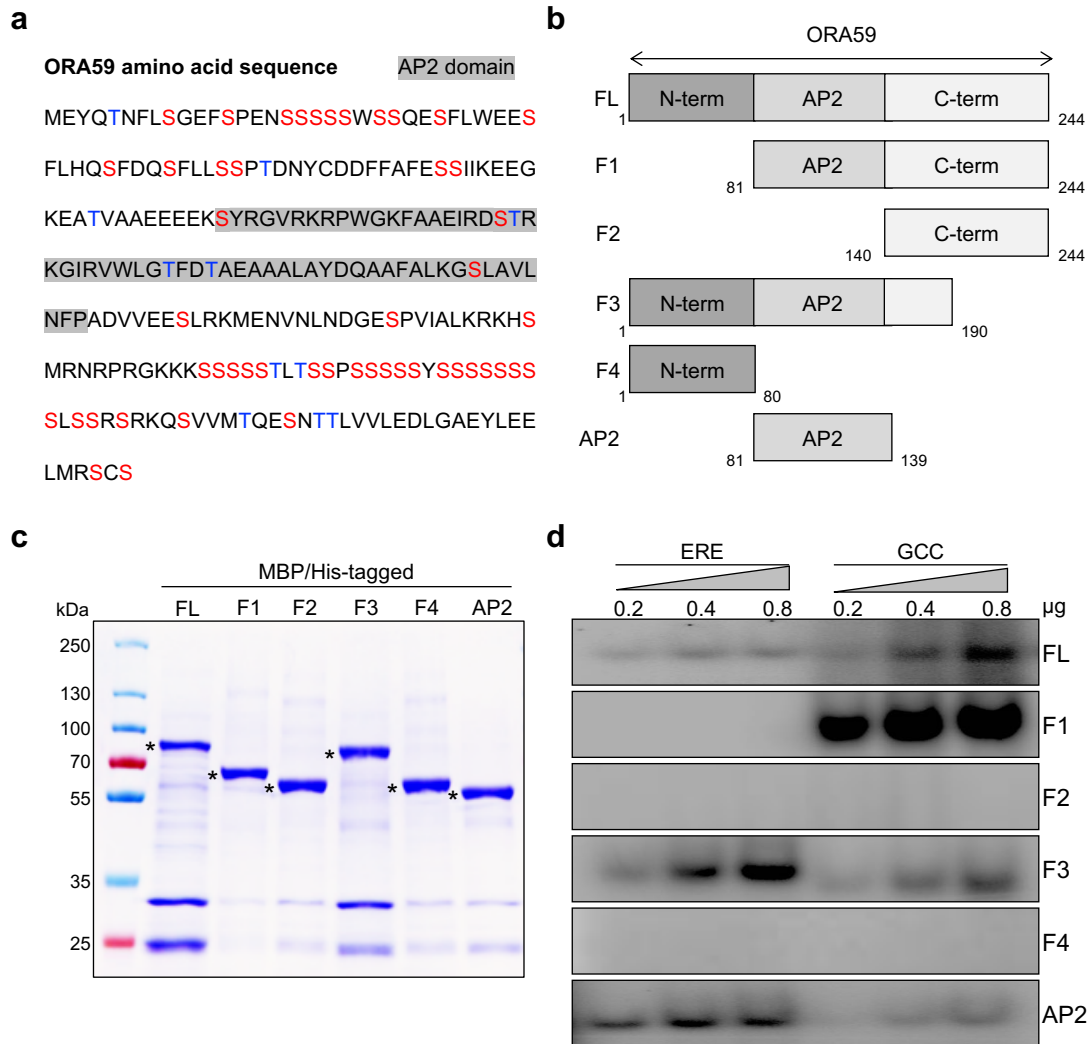


Figure 4. Truncated forms of ORA59 show distinct binding to ERE and GCC box.

a, Amino acid sequence of ORA59 protein. Ser and Thr residues are indicated in red and blue, respectively. The AP2 domain is shaded in gray. **b**, Schematic diagram of full-length and truncated ORA59 proteins prepared for EMSA. **c**, Coomassie blue staining of purified recombinant full-length and truncated ORA59 fused with N-terminal MBP and His tags. Asterisks indicate the corresponding purified proteins. **d**, DNA binding of different forms of ORA59 to the ERE and GCC box elements. Increasing amounts (0.2, 0.4, and 0.8 μg) of recombinant proteins were incubated with biotin-labeled ERE and GCC box oligonucleotide probes in EMSA. FL, full-length; GCC, GCC box.

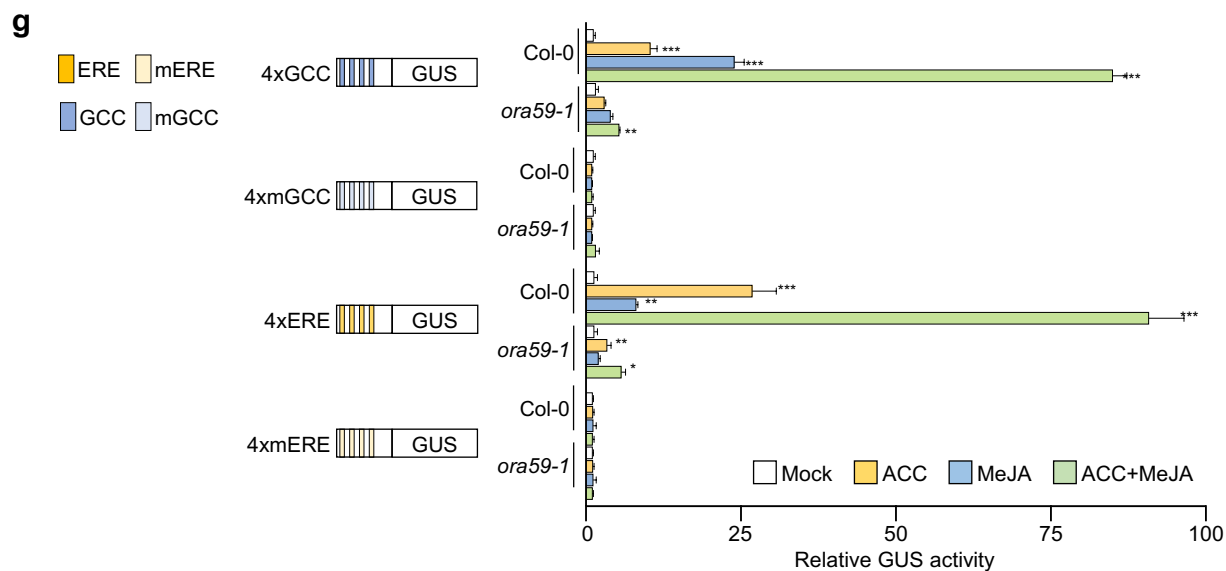
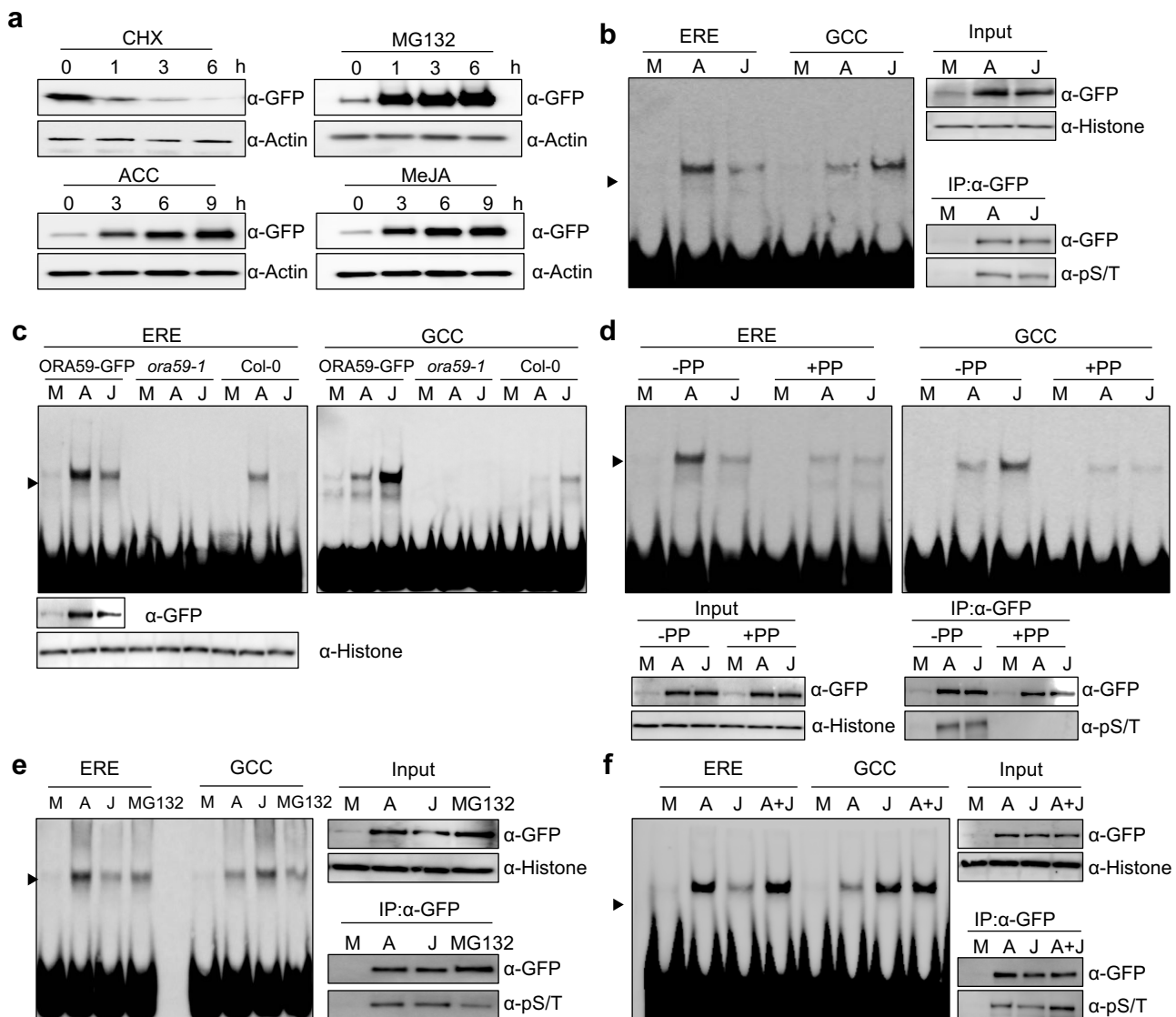


Figure 5. ORA59 binding to ERE and GCC box is regulated in ACC- and JA-dependent manners.

a, Immunoblot analysis of ORA59 stability in *35S:ORA59-GFP* plants. Six-week-old plants were treated with cycloheximide (100 μ M), MG132 (50 μ M), ACC (1 mM), and MeJA (100 μ M) for the indicated times. Protein extracts were subjected to immunoblotting with anti-GFP and anti-Actin antibodies. Actin levels served as a control. CHX, cycloheximide. **b**, EMSA analysis of nuclear extracts from *35S:ORA59-GFP* plants. Six-week-old plants were treated with ACC (1 mM) and MeJA (100 μ M) for 6 h. Nuclear extracts were incubated with biotin-labeled ERE and GCC box oligonucleotide probes in EMSA. **c**, EMSA analysis of nuclear extracts from *35S:ORA59-GFP*, *ora59*, and Col-0 plants. Six-week-old plants were treated with ACC (1 mM) and MeJA (100 μ M) for 6 h. Nuclear extracts were incubated with biotin-labeled ERE and GCC box oligonucleotide probes in EMSA. **d**, Effect of phosphatase treatment on DNA binding of ORA59. Six-week-old plants were treated with ACC (1 mM) and MeJA (100 μ M) for 6 h. Nuclear extracts were treated with lambda phosphatase for 30 min before incubation with biotin-labeled ERE and GCC box oligonucleotide probes in EMSA. **e**, Effect of MG132 treatment on DNA binding of ORA59. Six-week-old plants were treated with ACC (1 mM), MeJA (100 μ M), and MG132 (50 μ M) for 6 h. Nuclear extracts were incubated with biotin-labeled ERE and GCC box oligonucleotide probes in EMSA. **f**, Effect of ACC and MeJA co-treatments on DNA binding of ORA59. Six-week-old plants were treated with ACC (1 mM), MeJA (100 μ M), and a combination of ACC (1 mM) and MeJA (100 μ M) for 6 h. Nuclear extracts were incubated with biotin-labeled ERE and GCC box oligonucleotide probes in EMSA. **g**, GUS reporter assays showing the effect of ACC and MeJA co-treatments on the expression of the *GUS* reporter gene driven by synthetic promoters of 4xERE and 4xRAV, and their mutant versions 4xmERE and 4xmRAV. The left panel illustrates synthetic promoters. Transfected Col-0 and *ora59* protoplasts were treated with mock (water), ACC (200 μ M), MeJA (20 μ M), and a combination of ACC (200 μ M) and MeJA (20 μ M) for 6 h. Values represent means \pm SD ($n = 3$ biological replicates). Asterisks indicate significant differences from mock treatment as determined by one-way ANOVA with Tukey test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). In **b-f**, ORA59 levels (input) in nuclear extracts were determined by immunoblotting with anti-GFP and anti-Histone H3 antibodies. Histone levels served as a control. In **b,d-f**, to assess the phosphorylation status of ORA59, nuclear extracts were incubated with an anti-GFP antibody and the immunoprecipitated ORA59-GFP proteins were subjected to immunoblotting with anti-GFP and anti-phospho-Ser/Thr (pS/T) antibodies. IP, immunoprecipitation; GCC, GCC box; PP, phosphatase; A, ACC; J, MeJA.

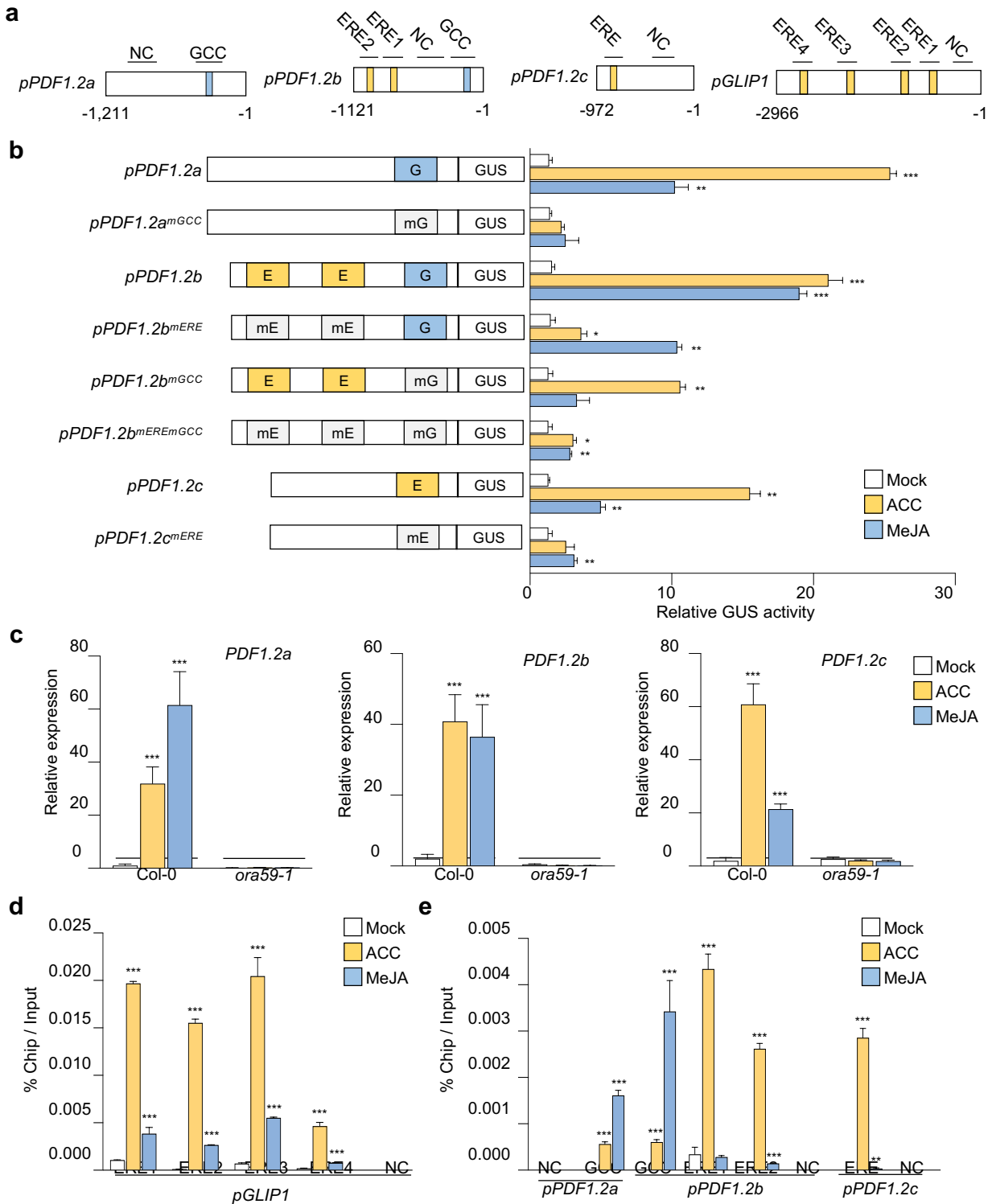


Figure 6. ORA59 directly binds to ERE and GCC box of *GLIP1* and *PDF1.2* promoters in ACC- and JA-dependent manners.

a, Schematic diagram of the ERE and GCC box elements in *PDF1.2* and *GLIP1* promoters. **b**, GUS reporter assays showing the ACC- and MeJA-induced expression of the *GUS* reporter gene driven by native or ERE/GCC box-mutated *PDF1.2a*, *b*, and *c* promoters. The left panel illustrates ERE and GCC box mutations of *PDF1.2a*, *b*, and *c* promoters. E, ERE; G, GCC box; mE, mutated ERE; mG, mutated GCC box. Transfected protoplasts were treated with mock (water), ACC (200 μ M), and MeJA (20 μ M) for 6 h. **c**, Analysis of *PDF1.2a*, *b*, and *c* expression in ACC- and MeJA-treated plants. **d,e**, ChIP-qPCR analysis for *in vivo* binding of ORA59 to ERE and GCC box sequences in the *GLIP1* (**d**) and *PDF1.2* (**e**) promoters. Chromatins from ACC- and MeJA-treated 35S:*ORA59-GFP* leaves were immunoprecipitated with an anti-GFP antibody. The enrichment of target element sequences is displayed as the percentage of input DNA. In **c-e**, six-week-old plants were treated with ACC (1 mM) and MeJA (100 μ M) for 6 h. NC indicates the negative control region without ERE and GCC box sequences. Values represent means \pm SD ($n = 3$ biological replicates). Asterisks indicate significant differences from mock treatment as determined by one-way ANOVA with Tukey test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

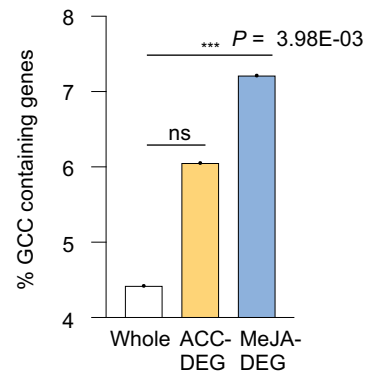
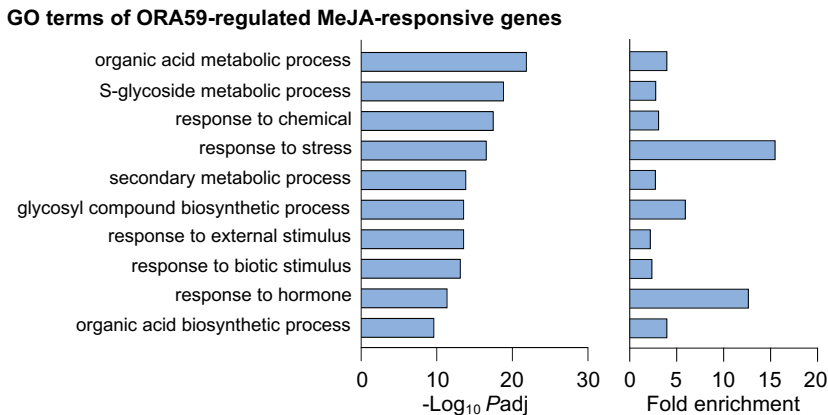
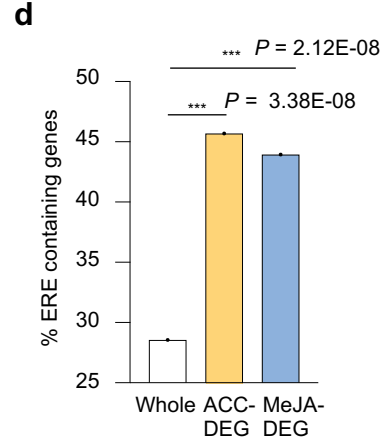
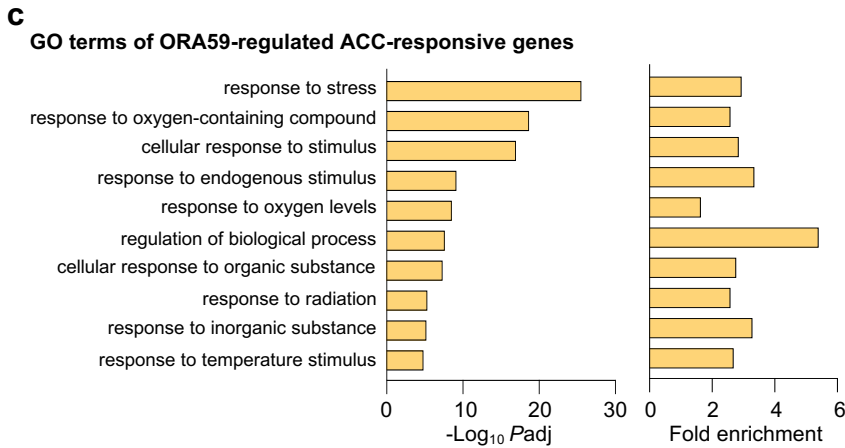
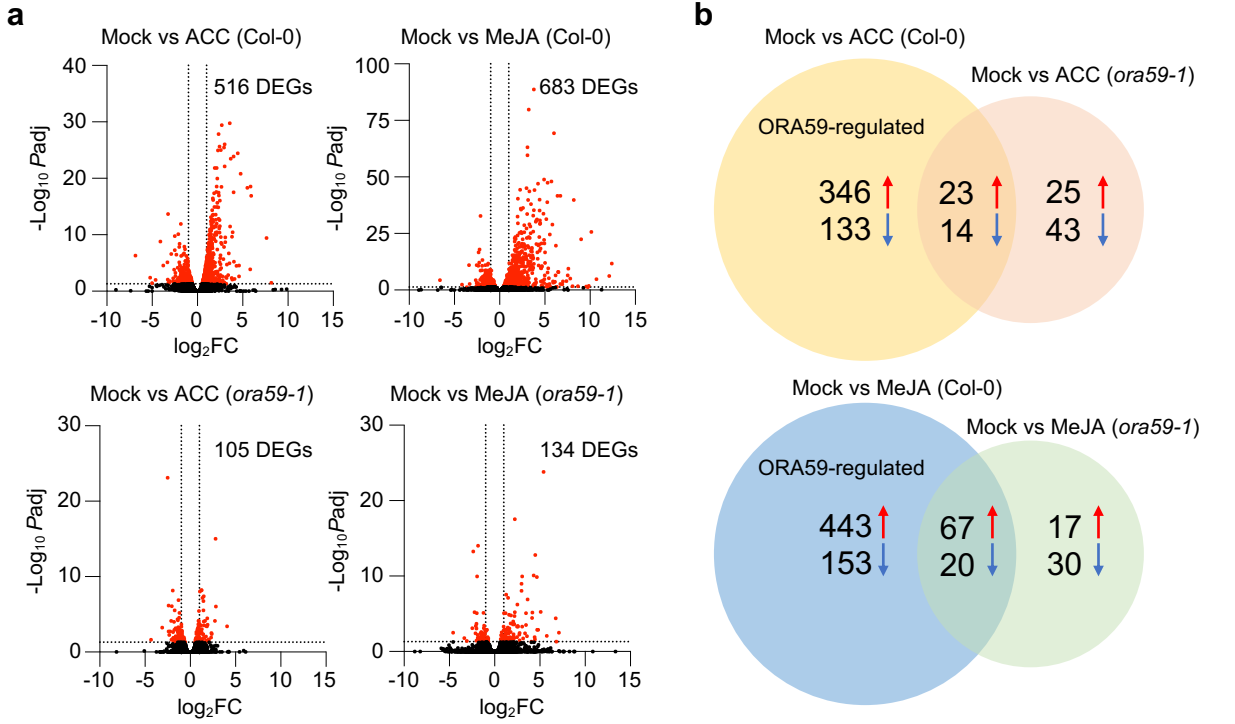


Figure 7. Identification ORA59-regulated ET- and JA-responsive genes by RNA-seq analysis.

a, Volcano plots of DEGs between mock and ACC/MeJA treatments in Col-0 and *ora59* plants. Cutoff values ($P_{adj} = 0.05$ and $|\log_2 FC| = 1$) are indicated by dashed lines. The red dots represent significantly upregulated and downregulated DEGs. **b**, Venn diagram of upregulated and downregulated DEGs between mock and ACC/MeJA treatments in Col-0 and *ora59* plants. **c**, GO enrichment analysis of ORA59-regulated DEGs. The 10 most significantly ($FDR < 0.05$) enriched GO terms in the Biological Process are presented for ACC- and MeJA-responsive genes. **d**, Analysis of the occurrence and enrichment of ERE and GCC box in promoters of ORA59-regulated ACC- and MeJA-responsive genes relative to whole *Arabidopsis* genes. The occurrence of ERE (AWTTCAAA) and GCC box (GCCGCC) sequences was analyzed using the regulatory sequence analysis tool (RSAT). Statistical significance of enrichment was determined by Fisher exact test ($***P < 0.001$). Whole, whole *Arabidopsis* genes; ns, not significant.

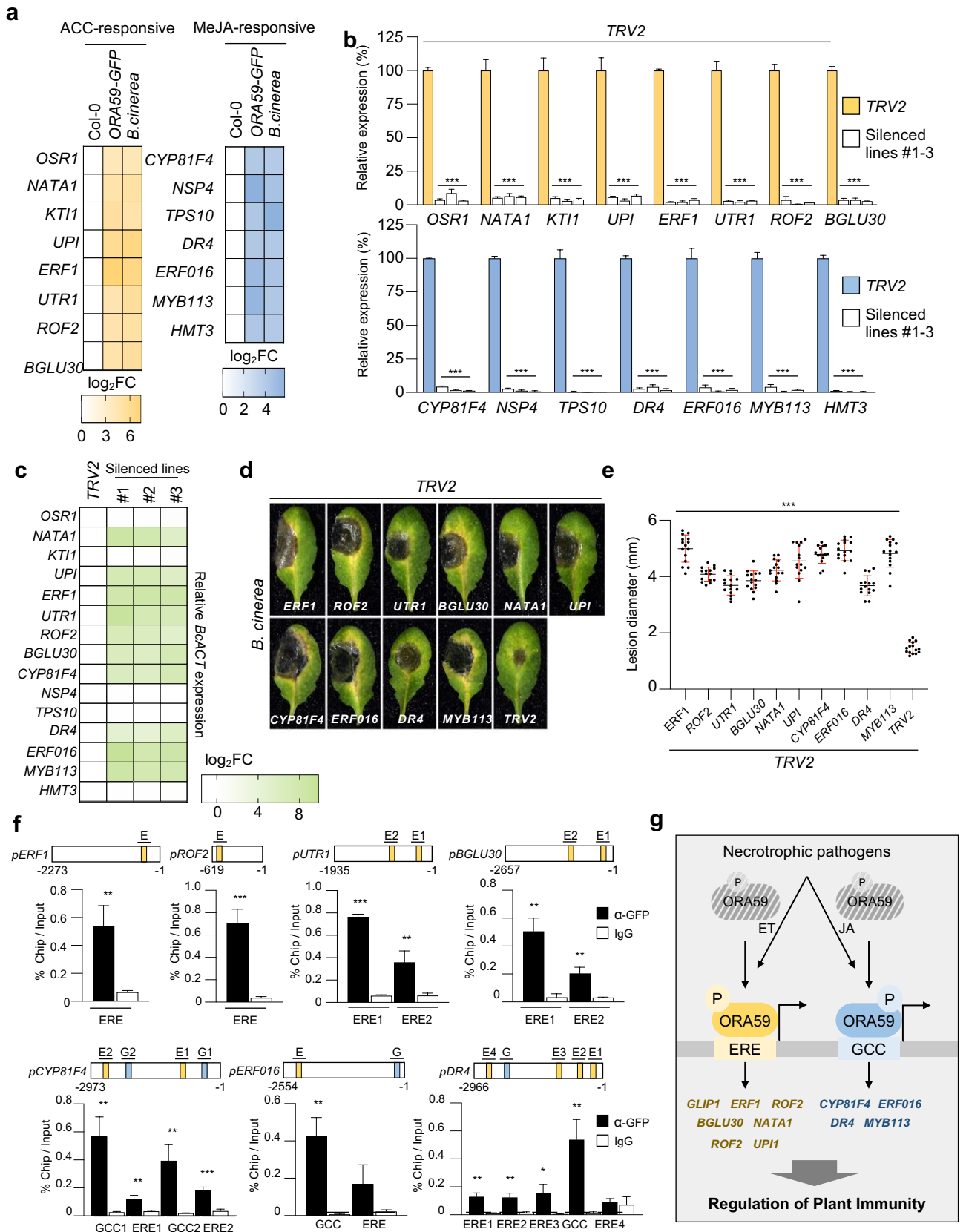


Figure 8. Identification of immunity-associated ORA59 target genes.

a, Heatmap showing the transcriptional levels of the most expressed DEGs in 35S:ORA59-GFP and *B. cinerea*-treated Col-0 plants relative to Col-0 plants. ACC-responsive eight ($|\log_2 \text{FC}| \geq 4$) and MeJA-responsive seven ($|\log_2 \text{FC}| \geq 4$) genes were selected for VIGS analysis. **b**, RT-qPCR analysis of the suppression of selected gene expression by VIGS. The transcript levels of selected genes in VIGS plants were determined relative to TRV2 control plants. Values represent means \pm SD ($n = 3$ biological replicates). **c**, Heatmap showing the abundance of *B. cinerea actin* (*BcACT*) gene relative to *Arabidopsis tubulin 2* (*AtTU2*) in *B. cinerea*-treated TRV2 control and VIGS plants. **d,e**, Disease symptoms (**d**) and lesion diameters (**e**) in *B. cinerea*-treated TRV2 control and VIGS leaves. Values represent means \pm SD ($n = 15$ infected leaves). **f**, ChIP-qPCR analysis for *in vivo* binding of ORA59 to ERE and GCC box sequences in ORA59 target gene promoters. Chromatins from 35S:ORA59-GFP leaves were immunoprecipitated with an anti-GFP antibody using pre-immune IgG as a negative control. The enrichment of target element sequences is displayed as the percentage of input DNA. Values represent means \pm SD ($n = 3$ biological replicates). E, ERE; G, GCC box. **g**, Model for the mechanism of ET/JA-responsive gene expression regulation by ORA59. Pathogenic infection triggers ET and JA biosynthesis, likely with different kinetic patterns, resulting in the activation of ET and JA signaling. These two hormone pathways lead to phosphorylation and stabilization of ORA59. ORA59 undergoes phosphorylation at different Ser/Thr residues in ET- and JA-dependent manners. This enhances DNA binding and transactivation activities of ORA59 with differential preference for ERE and GCC box. In this way, ET- and JA-activated ORA59 regulates different sets of genes and leads to fine-tuning of immune responses. In **c-e**, TRV2 control and VIGS plants were treated with 5 μl droplets of *B. cinerea* spore suspensions (5×10^5 spores ml^{-1}) for 2 days. Asterisks indicate significant differences from the TRV2 control (**b,e**) and the pre-immune IgG control (**f**) as determined by one-way ANOVA with Tukey test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).