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1 **Title: MicroProteins miP1b/BBX30 and miP1a/BBX31 form a positive feedback loop**
2 **with ABI5 to retard seedling establishment**
3

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15 **One sentence Summary:** Two microProteins stabilise and activate ABI5 to cause post-
16 germination growth arrest.

17

18 **Keywords:**

19 MicroProteins, BBX30, BBX31, ABI5, ABA(Abscisic acid), post-germination

20

21 **Author contributaion:**

22 SD and DS conceived the project. DS planned and performed all the experiments. SD wrote
23 the manuscript with help from DS. DS and SD analysed the data and helped preparing the
24 final manuscript.

25

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28 **Abstract:**
29 In plants the switch to autotrophic growth involves germination followed by post-germination
30 seedling establishment. When the environmental conditions are not favourable the stress
31 hormone Abscisic Acid (ABA) signals plants to postpone seedling establishment by inducing
32 the expression of the transcription factor ABI5. The levels of ABI5 determine the efficiency
33 of the ABA mediated post-germination developmental growth arrest. The molecular
34 mechanisms regulating the stability and activity of ABI5 during the transition to light is less
35 known. We found that two microProteins miP1a/ BBX31 and miP1b/BBX30 along with
36 ABI5 inhibit post-germination seedling establishment in a partially interdependent manner.
37 MicroProteins are single-domain proteins that interact with multi-domain proteins to
38 modulate their post-translational activity. miP1a/ BBX31 and miP1b/BBX30 physically
39 interact with ABI5 to stabilize it and promote its binding to promoters of downstream genes
40 in light. ABI5 reciprocally induces the expression of *BBX30* and *BBX31* by directly binding
41 to their promoter. ABI5 and the two microProteins thereby form a positive feedback loop to
42 promote ABA-mediated developmental arrest of seedlings. Our study highlights the
43 functional versatility of microProteins which can act as regulators of crucial developmental
44 transitions in plants as well as other eukaryotes.
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72 **Introduction:**

73 Growth is a fundamental characteristic of all living organisms. However, organisms invest
74 energy in growth only after their survival is ensured. In seed plants, just after germination,
75 the seed needs to make a crucial decision whether to proceed with seedling development or
76 not, based on the environmental conditions. This decision involves an interplay of signals
77 modulated by endogenous hormones and external factors. A post-germination developmental
78 checkpoint arrests the growth of seedlings when conditions are not favorable. One of the key
79 phytohormones that regulates germination and early plant development is Abscisic Acid
80 (ABA) (Weitbrecht et al., 2011; Lopez-Molina et al., 2001). It is also known as the stress
81 hormone that inhibits plant growth during times of stress. Among the external factors that
82 regulate seed germination and post-germination development, light plays a crucial role (Xu et
83 al., 2014). The role of ABA and light in regulating germination, indicated by the emergence
84 of the radicle, is quite well studied. Seedling establishment, as determined by the presence of
85 open green cotyledons, has traditionally been studied as part of the germination process.
86 However, several studies indicate that these two consecutive steps are developmentally
87 distinct and regulated by common as well as unique factors (Lopez-Molina et al., 2001).
88 Light promotes the autotrophic establishment of seedlings (Neff and Volkenburgh, 1994;
89 Chattopadhyay et al., 1998; Deng et al., 1991; Chen et al., 2008a). The molecular
90 understanding of the interplay between ABA and light to regulate post-germination seedling
91 establishment is limited.

92 Abiotic stress signals induce the accumulation of ABA by elevating its biosynthesis and
93 inhibiting its catabolism. ABA-deficient mutants germinate and establish faster than
94 wildtype, whereas ABA catabolism mutants accumulate more ABA and exhibit longer
95 dormancy periods (Tuteja, 2007; Xiong et al., 2002; Shu et al., 2018). ABA-induced by stress
96 is perceived and channeled through a signaling pathway to induce changes in gene expression
97 that ultimately lead to different stress responses. Studies utilizing the exogenous application
98 of ABA have allowed us to understand better the ABA signaling pathway and ABA-induced
99 responses. The ABA-signaling pathway involves the binding of ABA to PYRABACTIN
100 RESISTANCE1 (PYR)/ PYR1-LIKE (PYL)/ REGULATORY COMPONENTS OF ABA
101 RECEPTORS (RCAR) receptors leading to its interaction with type 2C protein phosphatases
102 (PP2Cs). This interaction and retention of PP2Cs by ABA-bound receptors leads to the
103 phosphorylation and release of the kinases SnRK2s, which further phosphorylate downstream
104 transcription factors inducing ABA-mediated gene expression (Ali et al., 2020). Numerous
105 transcription factors in Arabidopsis have been characterized for their roles in ABA signaling
106 and response. The bZIP transcription factor ABSCISIC ACID INSESNITIVE5 (ABI5) was
107 identified in a forward genetic screen for mutants exhibiting ABA insensitivity during
108 germination (Finkelstein and Lynch, 2000). Although ABI5 regulates the ABA-mediated
109 inhibition of germination, it plays a relatively more crucial role in desiccation tolerance and
110 ABA sensitivity during post-germination development (Maia et al., 2014). ABI5 promotes
111 post-germination arrest of seedlings to inhibit their growth and establishment under stress
112 (Lopez-Molina et al., 2001). It binds to the G-box type ABA response elements (ABRE) on
113 the promoters of several target genes including its promoter. EARLY METHIONINE-
114 LABELLED 1 (EM1) and EM6, which code for LATE EMBRYOGENESIS ABUNDANT

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115 (LEA) proteins, are some of the key downstream targets of ABI5 (Choi et al., 2000;
116 Finkelstein and Lynch, 2000; Carles et al., 2002).
117 Sensitivity to ABA during post-germination seedling development is substantially modulated
118 by light (Yadukrishnan and Datta, 2021). The interactions between the ABA and the light
119 signaling pathway components are reciprocal and multi-layered. ABI5 forms the point of
120 convergence of interactions with light signaling factors like PHYTOCHROME-
121 INTERACTING FACTORs (PIFs), ELONGATED HYPOCOTYL5 (HY5), FAR-RED
122 ELONGATED HYPOCOTYLS3 (FHY3), DE-ETIOLATED 1 (DET1), and B-box (BBX)
123 proteins at the transcriptional level (Yadukrishnan and Datta, 2021). In the dark, the PIF
124 proteins – PIF1, PIF3, PIF4, and PIF5 – directly bind to the *ABI5* promoter and induce its
125 transcription (Qi et al., 2020) . Additionally, ABI5 physically interacts with PIF1 and
126 enhances the binding of PIF1 onto the promoters of common target genes (Kim et al., 2016).
127 The transcription factor HY5 also directly binds to the promoter of *ABI5* and promotes its
128 expression (Chen et al., 2008a). It was recently identified that HY5 acts downstream of COP1
129 to negatively regulate ABA-mediated inhibition of postgermination seedling development
130 (Yadukrishnan et al., 2020). FHY3 and DET1 directly bind to the *ABI5* promoter to activate
131 or suppress its expression respectively and thereby optimize seedling greening during early
132 development (Tang et al., 2013). In darkness, DET1 stabilizes the PIF proteins to indirectly
133 enhance *ABI5* expression (Dong et al., 2014). BBX proteins are B-box containing zinc finger
134 transcription factors that regulate several aspects of light-mediated development including
135 photomorphogenesis, flowering, shade avoidance, high light, and UV-B tolerance (Gangappa
136 and Botto, 2014; Xu et al., 2014). Some BBX proteins play crucial roles in the light-ABA
137 signaling crosstalk (Vaishak et al., 2019). BBX21 is a transcriptional activator of *HY5* and a
138 positive regulator of photomorphogenesis (Datta et al., 2007; Xu et al., 2014). BBX21
139 physically interacts with HY5 and inhibits its binding on the *ABI5* promoter (Xu et al., 2014)
140 . BBX21 also interacts with ABI5 and prevents it from binding on its own promoter, thereby
141 reducing *ABI5* expression (Xu et al., 2014). Moreover, BBX21 directly binds to the *ABI5*
142 promoter and recruits a chromatin remodeler protein HRB2 (HYPERSENSITIVE TO RED
143 AND BLUE 2) to alter the chromatin structure on the *ABI5* promoter and reduce *ABI5*
144 transcription (Kang et al., 2018). Another B-box protein BBX19 binds to the promoter of
145 *ABI5* and induces its expression to promote ABA-mediated inhibition of germination and
146 seedling development (Bai et al., 2019). CO and COL4/BBX5 also regulate ABA signaling,
147 although the mechanistic details need further investigation (Min et al., 2015)
148 ABI5 function is tightly regulated post-translationally by several factors. The activity of
149 ABI5 is turned on by SnRK2s through phosphorylation, while it gets deactivated through
150 dephosphorylation by PP2Cs (Nakashima et al., 2009). Furthermore, its activity is fine-tuned
151 through modifications such as ubiquitination, sumoylation, and S-nitrosylation (Stone et al.,
152 2006; Albertos et al., 2015; Miura and Hasegawa, 2009).The stability and activity of ABI5
153 protein are also modulated by components of the light signaling pathway like COP1. *cop1*
154 shows ABA hyposensitivity during post-germination seedling development (Yadukrishnan et
155 al., 2020). COP1 mediates ABA-induced accumulation of ABI5 by physically interacting
156 with ABA-hypersensitive DCAF1 (ABD1) that targets ABI5 for degradation. COP1
157 ubiquitinates ABD1 to promote its degradation and thereby enhances ABI5 protein stability
158 in dark (Peng et al., 2022). Additionally, COP1 promotes the binding of ABI5 to its target

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159 promoters to inhibit seedling growth (Yadukrishnan et al., 2020). Regulation by
160 microProteins has emerged as a relatively new mechanism of post-translational control of
161 protein abundance and activity in various organisms (Rodrigues et al., 2021; Kruusvee et al.,
162 2022; Kruusvee and Wenkel, 2022). MicroProteins are small, single-domain proteins
163 generally less than 140 amino acids long that share sequence homology with multi-domain
164 proteins (Hong et al., 2005; Dolde et al., 2018). ABI5 inhibits seedling establishment under
165 unfavorable conditions when a seedling tries to emerge into the light from the darkness
166 underneath the soil. The efficacy of this post-germination developmental growth arrest
167 depends on the levels of ABI5 (Lopez-Molina et al., 2003). How the stability of ABI5 is
168 regulated, especially under light, and factors modulating its activity is relatively less
169 understood.
170 Here we found that the two light inducible microProteins BBX31/miP1a and BBX30/miP1b
171 and ABI5 arrest post-germination seedling development in an interdependent manner. ABI5
172 directly binds to the promoter of *BBX30* and *BBX31* and induces their transcription. BBX30
173 and BBX31 physically interact with ABI5 and promote its stabilization. BBX30 and BBX31
174 also enhance ABI5-mediated gene expression by promoting the binding of ABI5 on its target
175 promoters. Taken together our study suggests that ABI5 and the microProteins BBX30 and
176 BBX31 arrest seedling growth by a positive feedback mechanism.
177
178 **Results**
179 **BBX30 and BBX31 promote ABA-mediated inhibition of post-germination seedling**
180 **development**
181 *BBX30* and *BBX31* are well-characterized light-responsive genes playing roles in
182 photomorphogenesis, UV-B signaling, and flowering (Heng et al., 2019; Yadav et al., 2019;
183 Graeff et al., 2016). In one of our previous studies, we found that the expression of several
184 genes involved in ABA response and desiccation tolerance was upregulated in *35S:BBX31*
185 (Yadav et al., 2019). In another study, similar genes were downregulated in *bbx30bbx31*
186 (Heng et al., 2019). *BBBX30* and *BBX31* expression is also upregulated by ABA (Xu et al.,
187 2019). The promoter sequences of *BBX30* and *BBX31* contain Abscisic Acid (ABA) response
188 elements (ABRE). All of these prompted us to ask the question if these genes might be
189 regulated by ABA to modulate stress responses. We started studying seedling development,
190 considering the broad function of these microproteins in regulating early developmental
191 responses. We grew imbibed seeds of Col-0, *bbx30*, *bbx31*, *bbx30bbx31*, *BBX30OE*, and
192 *BBX31OE* on a half-strength MS plate, in the presence (+) or absence (-) of ABA. Seedlings
193 with open green cotyledons were used to calculate % seedling establishment. In (-) ABA
194 conditions we did not find significant difference in the % seedling establishment of Col-0,
195 *bbx30*, *bbx31*, *bbx30bbx31*, *BBX30OE*, and *BBX31OE*. Seedlings of all the genotypes
196 showed 100% seedling establishment by 3rd day after stratification (DAS) (Figure 1, A-C).
197 However, the % seedling establishment in +ABA conditions was higher in *bbx30, bbx31* and
198 *bbx30bbx31* and substantially lower in *BBX30OE* and *BBX31OE* compared to Col-0 (Figure
199 1, A, D and E). In order verify if the difference in seedling establishment rate is due to
200 differences in germination, we determind the % germination by counting seeds with emerged
201 radicle in (-) and (+) ABA conditions. However we did not find difference in % germination
202 among the genotypes in either (-) or (+) ABA containing plates. Seedlings of all the

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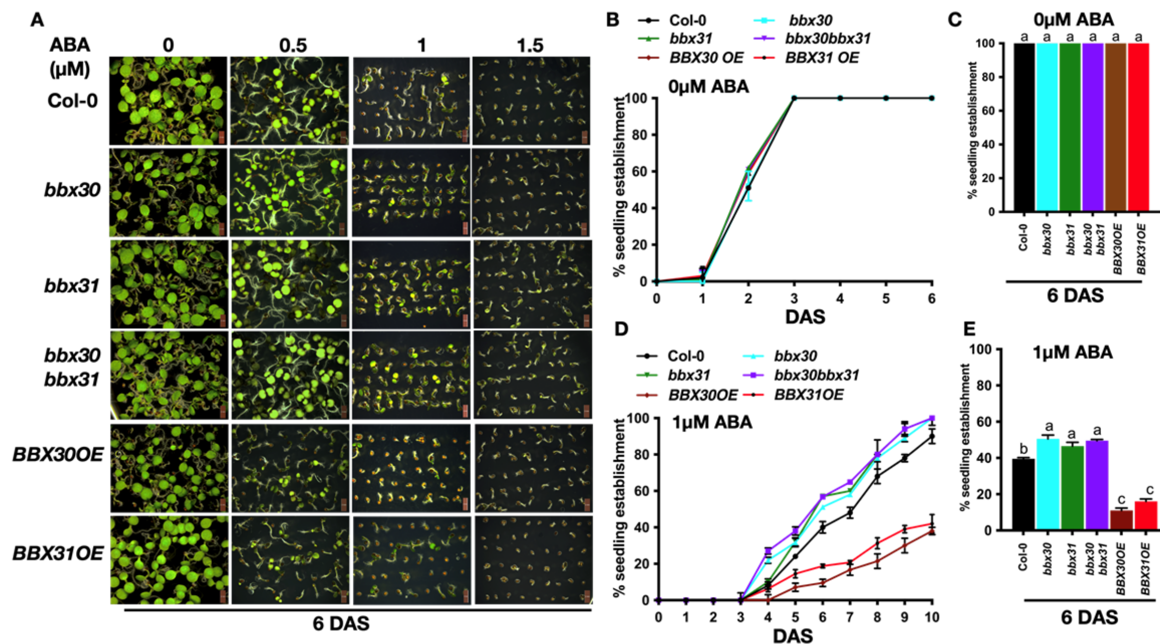
203 genotypes exhibit 100% germination by the 2nd day after stratification (DAS) in -ABA
204 conditions while total germination is achieved at 4 DAS under +ABA conditions
205 (Supplemental Figure S1, A-D). This suggests that BBX30 and BBX31 promote ABA-



206 mediated inhibition of seedling development at the post-germination stage.
207
208
209 **Figure 1. BBX30 and BBX31 promote ABA-mediated inhibition of post-germination**
210 **seedling development**
211 A, Representative images of 6-day-old seedlings of Col-0, *bbx30, bbx31*, *bbx30bbx31*,
212 *BBX30OE* and *BBX31OE* grown on a 0.5x MS plate supplemented with 0μM, 0.5μM, 1μM,
213 1.5μM of ABA. B, C. The percentage (%) of seedling establishment in -ABA (0μM)
214 conditions for the indicated genotypes for up to 6 days after stratification (DAS) and on day 6
215 (C). D, E, % seedling establishment in +ABA (1μM) conditions for the indicated genotypes
216 for up to 10 days after stratification (DAS)(D) and on day 6 (E). Seedlings with open, green
217 cotyledons were counted to determine % seedling establishment. In (B-E) Error bars
218 represent SEM of three independent experiments with >100 seeds per experiment. In (C) and
219 (E) letters above the bar indicate the statistical groups as determined by one-way ANOVA
220 followed by Tukey's *post hoc* test ($p < 0.05$).
221
222 **BBX30, BBX31 and ABI5 mediate post-germination seedling growth arrest in an inter**
223 **dependent manner**
224
225 The transcription factor ABI5 is a key regulator of several ABA responses including post-
226 germination development and is known to mediate interplay with light signaling factors
227 (Chen et al., 2008b; Yadukrishnan et al., 2020; Lopez-Molina et al., 2001). We asked the
228 question if ABI5 plays any role in BBX30 and BBX31 mediated seedling growth arrest. In
229 presence of ABA, % seedling establishment up to 8 DAS is higher in *abi5-8* compared to

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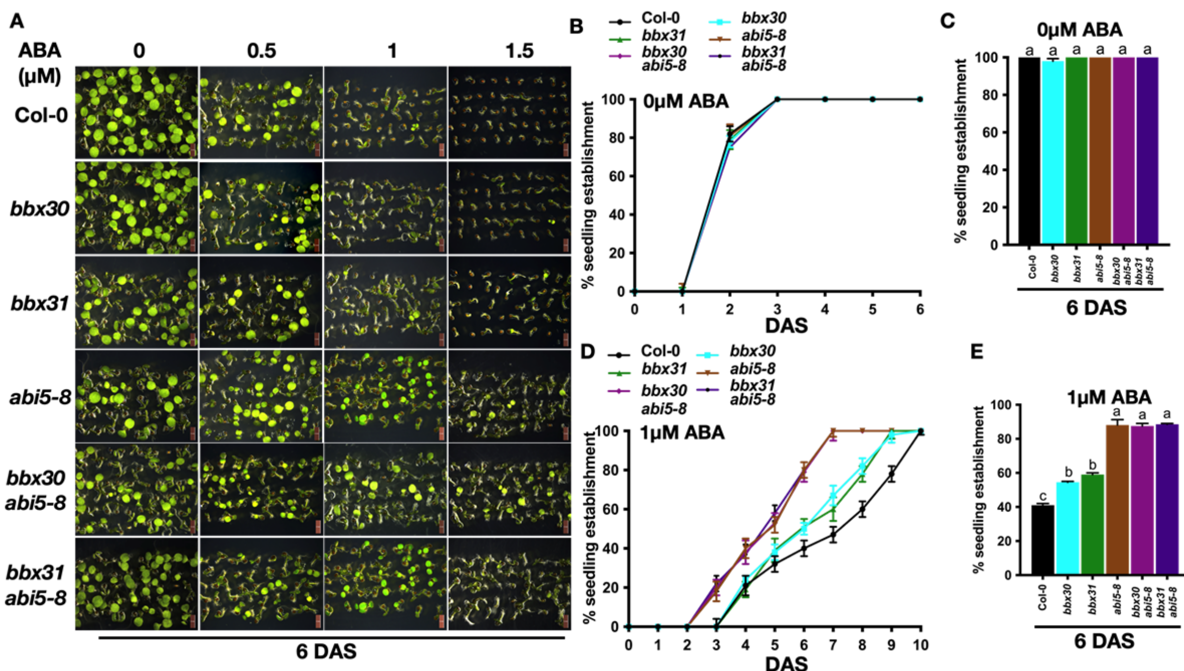
230 Col-0, *bbx30* and *bbx31* (Figure 2, A, D and E). This difference was not seen in -ABA
231 conditions (Figure 2, A, B and C). In order to examine the genetic interaction between
232 *BBX30*, *BBX31* and *ABI5* we generated *bbx30abi5-8* and *bbx31abi5-8* by crossing and
233 monitored the seedling establishment rates of the double mutants under ABA. The
234 establishment rates in *bbx30abi5-8,* and *bbx31abi5-8* were similar to *abi5-8* suggesting that
235 BBX30 and BBX31 regulate these processes in an ABI5-dependent fashion (Figure 2, A-E).
236 There was no difference in the % germination among these genotypes in (-) ABA conditions,
237 with all genotypes attaining 100% germination by 2 DAS (Supplemental Figure S2, A and
238 B). In (+) ABA conditions at 2 DAS % germination was higher in *abi5-8, bbx30abi5-8,* and
239 *bbx31abi5-8* compared to Col-0, *bbx30* and *bbx31*, however by 5 DAS there was no
240 significant difference in the germination % between the genotypes (Supplemental Figure S2,
241 C and D).
242



243

244 **Figure 2. *BBX30, BBX31* and *ABI5* interact genetically to regulate early development in**
245 **Arabidopsis**
246 A, Representative images of 6 day-old seedlings of Col-0, *bbx30, bbx31*, *abi5-8*, *abi5-*
247 *8bbx30*, *abi5-8bbx31* grown on a 0.5x MS plate supplemented with 0μM, 0.5μM, 1μM,
248 1.5μM of ABA. B, C, % seedling establishment in -ABA (0μM) conditions for the indicated
249 genotypes for up to 6 days after stratification (DAS) (B) and at day 6 (C). D, E, % seedling
250 establishment in +ABA (1μM) conditions for the indicated genotypes for up to 10 days after
251 stratification (DAS) (D) and at day 6 (E). Seedlings with open, green cotyledons were
252 counted to determine % seedling establishment. In (B-E) error bar represents SEM of three
253 independent experiments with >100 seeds per experiment. In (C) and (E) letters above the bar
254 indicate the statistical groups as determined by one-way ANOVA followed by Tukey's *post*
255 *hoc* test ($p < 0.05$).
256

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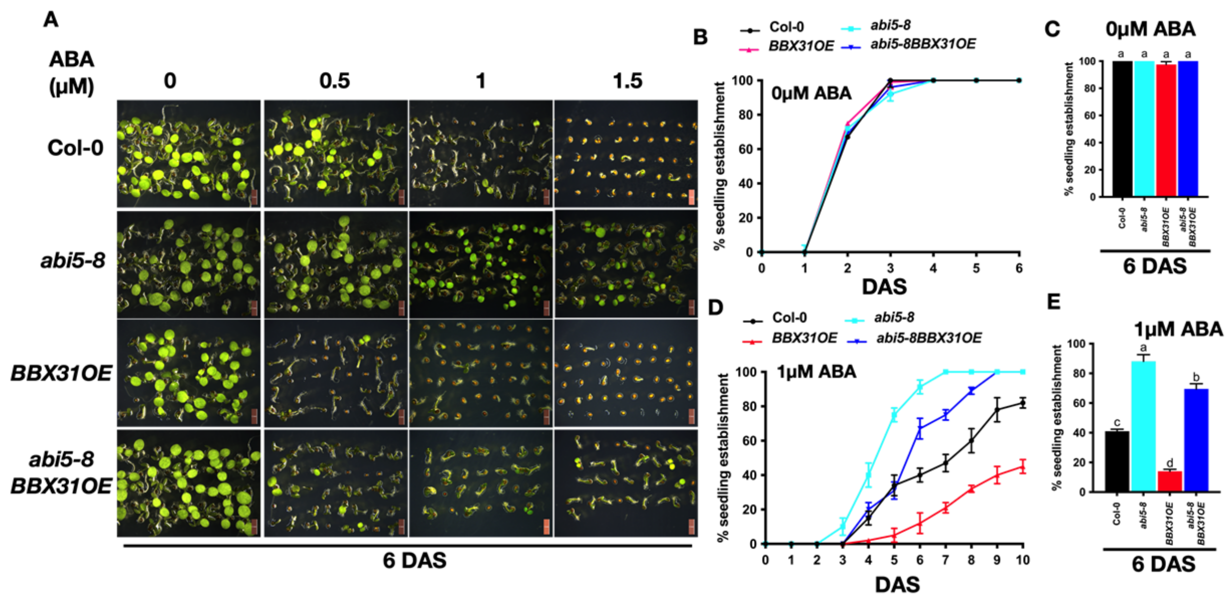
257 We further crossed *BBX31OE* with *abi5-8* to obtain *abi5-8BBX31OE* and studied their ABA
258 sensitivity during early seedling development (Figure 3A). There is no significant difference
259 in the germination rates of Col-0, *abi5-8, BBX31OE* and *abi5-8BBX31OE* by 5 DAS in
260 presence or absence of ABA (Supplemental Figure S3, A and B). However, at 6 DAS under
261 1µM ABA, *abi5-8* and *BBX31OE* exhibit 85% and 15% seedling establishment respectively
262 as compared to 40% in Col-0 (Figure 3, D and E). Seedling establishment % in *abi5-*
263 *8BBX31OE* is around 70% (Figure 3, D and E). There is no difference in the % seedling
264 establishment between the genotypes under 0µM ABA (Figure 3, B and C). These data
265 further validate that BBX31-mediated inhibition of post-germination seedling development is
266 at least partially ABI5 dependent.



267
268
269 **Figure 3. BBX31 regulates seedling growth arrest in an ABI5-dependent fashion**
270 A, Representative images of 6-day-old seedlings of Col-0*, abi5-8, BBX31OE, abi5-*
271 *8BBX31OE* grown on a 0.5x MS plate supplemented with 0µM, 0.5µM, 1µM, 1.5µM of
272 ABA. B, C, % seedling establishment in -ABA (0µM) conditions for the indicated genotypes
273 for up to 6 days after stratification (DAS) (B) and at day 6 (C). D, E, % seedling
274 establishment in +ABA (1µM) conditions for the indicated genotypes for up to 10 days after
275 stratification (DAS) (D) and at day 6 (E). Seedlings with open, green cotyledons were
276 counted to determine % seedling establishment. In (B-E) Error bars represent SEM of three
277 independent experiments with >100 seeds per experiment. In (C) and (E) letters above the bar
278 indicate the statistical groups as determined by one-way ANOVA followed by Tukey's *post*
279 *hoc* test ($p < 0.05$).
280
281 Next, we set up genetic crosses of *ABI5* overexpression line (*ABI5OE*) with *bbx30*, *bbx31*,
282 and *bbx30bbx31* to generate *bbx30ABI5OE*, *bbx31ABI5OE*, and *bbx30bbx31ABI5OE* lines,
283 respectively. Under 0µM ABA, germination and seedling establishment of all the genotypes
284 progressed at similar rates till attaining 100% (Figure 4, B and C, Supplemental Figure 4, A

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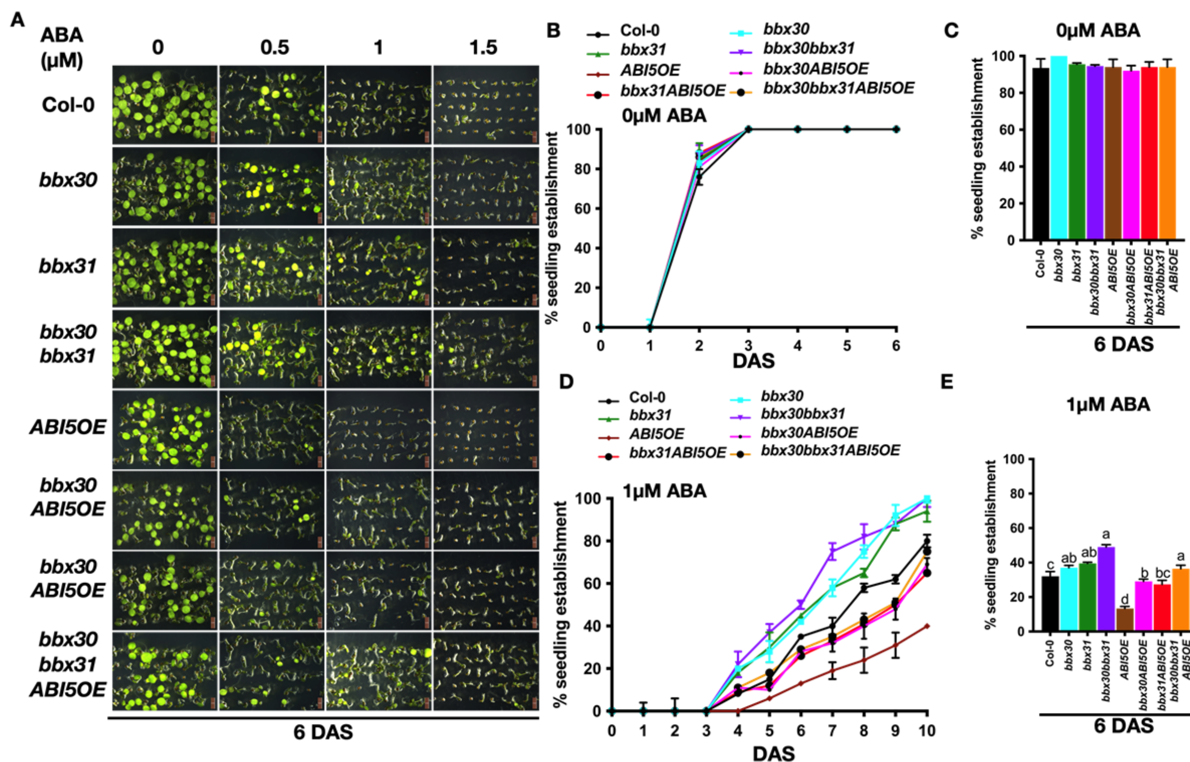
285 and B). In presence of 1µM ABA, *ABI5OE* exhibited lower rate of seedling establishment as
286 compared to the wildtype (Figure 4, D and E). Interestingly, *bbx30ABI5OE*, *bbx31ABI5OE,*
287 and *bbx30bbx31ABI5OE* lines showed higher seedling establishment compared to *ABI5OE*
288 (Figure 4, D and E). We observed similar growth pattern among the genotypes in 0.5 and 1.5
289 µm ABA ((Figure 4, A). In 1µM ABA, *ABI5OE* showed lower % germination compared to
290 Col-0, which was partially complemented in *bbx30ABI5OE*, *bbx31ABI5OE*, and
291 *bbx30bbx31ABI5OE* lines at 2 DAS (Supplemental Figure 4, C and D). All these findings
292 together suggest that the BBX proteins BBX30 and BBX31 are required for ABI5-mediated
293 seedling growth arrest. This further suggests that the dependence of ABI5 on BBX30 and
294 BBX31 is probably at the protein level, as the reduced seedling establishment phenotype of
295 seedlings constitutively expressing *ABI5* is partially rescued by the loss of *BBX30* and
296 *BBX31*.
297



298
299
300 **Figure 4. BBX30 and BBX31 are required for ABI5-mediated seedling growth arrest**
301
302 A, Representative images of 6-day-old seedlings of Col-0*, bbx30, bbx31*, *bbx30bbx31,*
303 *ABI5OE*, *bbx30ABI5OE, bbx31ABI5OE*, *bbx30bbx31ABI5OE* grown on a 0.5x MS plate
304 supplemented with 0µM, 0.5µM, 1µM, 1.5µM of ABA. B, C, % seedling establishment in -
305 ABA (0µM) conditions for the indicated genotypes for up to 6 days after stratification (DAS)
306 (B) and at day 6 (C). D, E, % seedling establishment in +ABA (1µM) conditions for the
307 indicated genotypes for up to 10 days after stratification (DAS) (D) and at day 6 (E).
308 Seedlings with open, green cotyledons were counted to determine % seedling establishment.

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309 In (B-E) Error bars represent SEM of three independent experiments with >100 seeds per
310 experiment. In (C) and (E) letters above the bar indicate the statistical groups as determined
311 by one-way ANOVA followed by Tukey's *post hoc* test ($p < 0.05$).
312
313 **ABI5 physically interacts with BBX30 and BBX31**
314
315 Since constitutive overexpression of *ABI5* could not enhance ABA response in the absence of
316 *BBX30* and *BBX31*, we hypothesized that BBX30 and BBX31 might interact with ABI5
317 protein and regulate its activity. In order to confirm physical interaction between ABI5 and
318 the BBX proteins be used yeast two-hybrid, in vitro pull down and BiFC assays. Our yeast-
319 two-hybrid assay using ABI5 as bait and either BBX30 or BBX31 as prey indicated that
320 ABI5 can physically interact with both BBX30 and BBX31 (Figure 5, A). According to
321 previous studies ABI5 contains conserved domains, that govern specific functions (Lopez-
322 Molina et al., 2003; Finkelstein and Lynch, 2000). We examined the interaction of BBX31
323 with different domains of ABI5, and observed that the conserved C1 domain of ABI5 is
324 required for the interaction with the N-terminal of BBX31 (Supplemental Figure 5, A and B).
325 To further confirm the interaction between BBX30, BBX311 and ABI5, we performed in-
326 vitro pull-down experiment using full length ABI5 fused to GST, and BBX30 and BBX31
327 proteins fused to 6xHis epitope. GST-ABI5 was able to pull down His-tagged BBX30 and
328 BBX31, validating that BBX30 and BBX31 physically interact with ABI5 in vitro (Figure 5,
329 B). We also validated the interaction of these proteins in vivo using bimolecular fluorescence
330 complementation (BiFC) in *Nicotiana benthamiana* (Figure 5C). All these data together
331 suggest that ABI5 physically interacts with BBX30 and BBX31.

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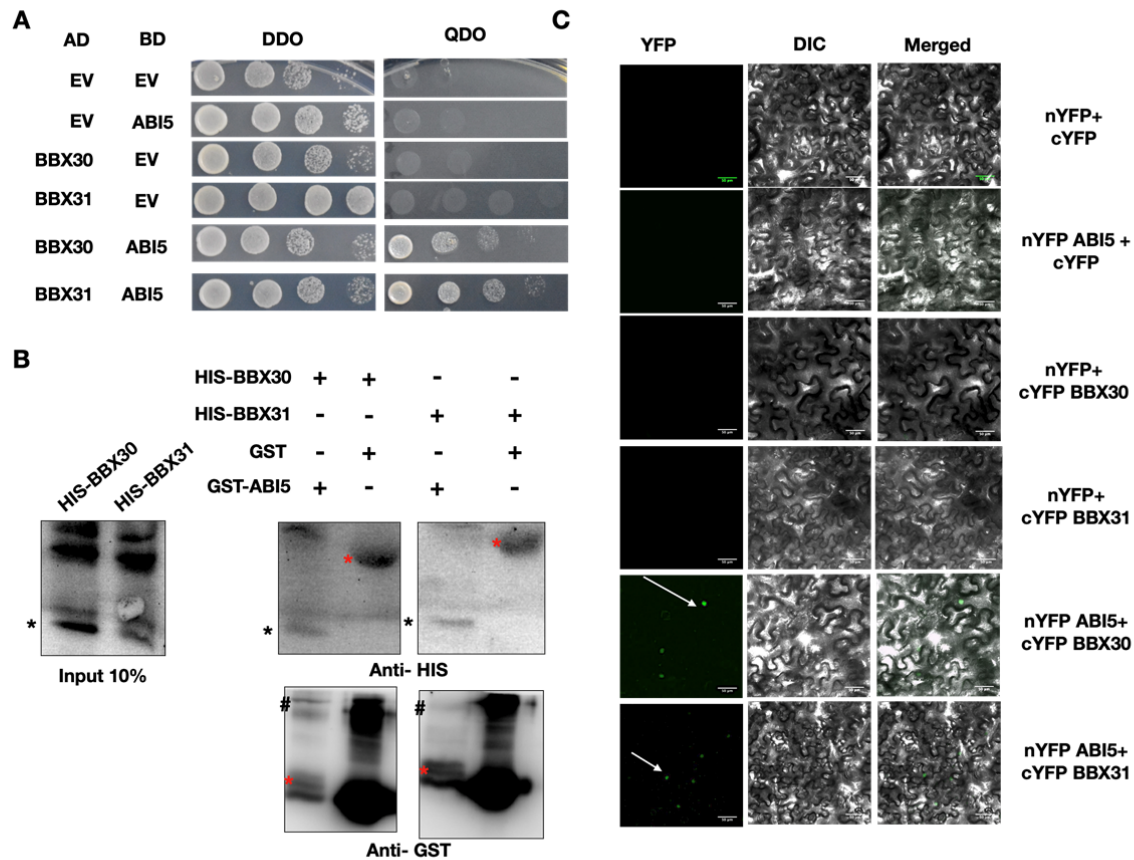

332
333
334
335 **Figure 5. ABI5 physically interacts with BBX30 and BBX31**
336 A, Yeast two-hybrid assay showing the interaction between BBX30, BBX31 and ABI5.
337 pGADT7 (AD) and pGBKT7 (BD) represent the GAL4 activation domain and binding
338 domain respectively. EV indicates empty AD or BD vector. BBX30 and BBX31 were fused
339 to AD, while ABI5 was fused with BD and tested for interaction. DDO represents the
340 medium lacking leucine and tryptophan, while QDO additionally lacks histidine and adenine.
341 B, In-vitro pull-down assay showing the interaction between BBX30, BBX31 with ABI5.
342 BBX30 and BBX31 were fused with N-terminal HIS tag, while ABI5 is fused with N
343 terminal GST tag. To check the physical interaction bead-bound GST and GST-ABI5 were
344 used to pull down His-BBX30 and His-BBX31. Precipitated proteins were analysed by anti-
345 GST and anti-His antibodies. * mark indicate band specific to BBX30 and BBX31, #
346 indicates GST-ABI5 corresponding band, *(red) indicate non-specific band. C, BiFC assay
347 showing the interaction of BBX30 and BBX31 with ABI5 in planta. nYFP and cYFP
348 represent the N-terminal and C-terminal half of yellow fluorescent protein. Vectors in the
349 indicated combinations were transformed into Agrobacterium and co-infiltrated into the 3-
350 weeks-old *N. benthamiana* leaves and imaged after 36 hours. The scale bar represents 50µm.
351
352 **BBX30 and BBX31 stabilize ABI5 during post-germination development**

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353
354 BBX30 and BBX31 belong to the group of microProteins that can sequester other proteins
355 and often regulate their stability (Wu et al., 2020; Graeff et al., 2016). Our protein-protein
356 interaction studies indicated that BBX30 and BBX31 physically interact with ABI5 (Figure
357 5). In order to understand the physiological significance of this interaction, we examined if
358 BBX30 and BBX31 can regulate ABI5 protein abundance. Initially we put Col-0 and
359 *bbx30bbx31* seeds on plates containing 0μM and 1μM ABA and harvested samples every day
360 for up to 4 days after stratification and determind ABI5 accumulation by immunoblotting
361 (Supplemental Figure 6, A and B). ABI5 accumulated to high levels on day 1 in both Col-0
362 and *bbx30bbx31*(Supplemental Figure 6, A and B). In -ABA conditions ABI5 accumulation
363 in day 2, 3 and 4 samples in both the genotypes was decreased (Supplemental Figure 6, A and
364 B). In presence of ABA, ABI5 accumulation was detected on day 2, 3 and 4 samples in Col-0
365 but not in *bbx30bbx31* (Supplemental Figure 6, A and B). This suggests that BBX30 and
366 BBX31 might regulate the stability of ABI5 especially during post-germination development.
367 To check the dynamics of ABI5 protein accumulation upon ABA treatment during post-
368 germination development, we treated germinated seeds of Col-0, *bbx30bbx31*, and *BBX31OE*
369 with 10μM ABA for 1, 6, and 12 hours and harvested samples to determine ABI5 protein
370 levels (Figure 6, A-D). ABI5 accumulation was lower in *bbx30bbx31* compared to Col-0,
371 whereas in *BBX31OE* enhanced accumulation of ABI5 was detected (Figure 6, A-D). Similar
372 ABA treatment causes a gradual increase in the ABI5 accumulation in *ABI5OE*, while this is
373 compromised in *bbx30 bbx31 ABI5OE*, suggesting that these BBX proteins promote ABA
374 mediated post-germination ABI5 accumulation (Figure 6E). Previous reports have shown that
375 ABI5 protein undergoes degradation via the 26S proteasomal pathway (Lee et al., 2010;
376 Stone et al., 2006). Inhibition of 26S proteasome pathway by treating the germinated seeds
377 with MG132 for 6 hours prevented the decrease of ABI5 levels in *bbx30bbx31*, suggesting
378 that BBX30 and BBX31 might promote ABI5 accumulation by negatively regulating its
379 degradation (Figure 6, F). To uncouple the effects of de novo translation on ABI5 levels in
380 these genotypes, we treated the germinated seeds with the translational inhibitor
381 cycloheximide (CHX) and examined the stabilization of ABI5 in Col-0*, bbx30bbx31 and*
382 *BBX31OE* (Figure 6, G and H). The rate of degradation of ABI5 was faster in *bbx30bbx31,*
383 while reduced in *BBX31OE* as compared to Col-0 (Figure 6, G and H). All these results
384 together suggest that BBX30 and BBX31 promote the stabilization of ABI5 during post-
385 germination development by negatively regulating its proteasome-mediated degradation.

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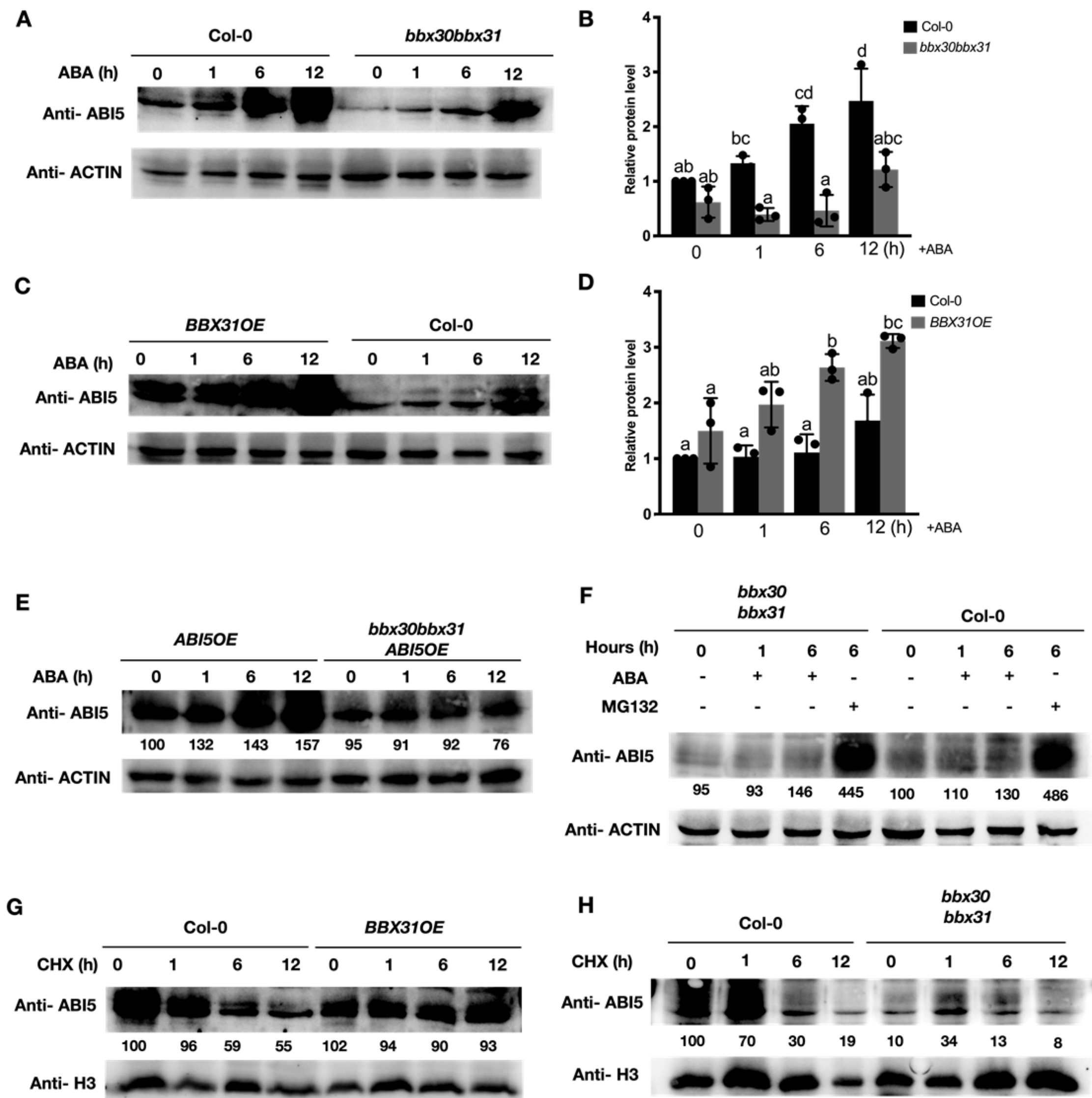

386
387
388 **Figure 6. BBX30 and BBX31 stabilize ABI5**

389 A, Immunoblot showing the abundance of ABI5 protein in Col-0 and *bbx30bbx31*. B,
390 Relative protein levels of ABI5 in Col-0 and *bbx30bbx31*. C, Immunoblot showing the
391 abundance of of ABI5 protein in *BBX31OE* and Col-0. D, Relative protein levels of ABI5 in
392 *BBX31OE* and Col-0. In (B) and (D) error bars represent standard deviation of two blots.
393 Letters above the error bar indicate the statistical groups as determined by one-way ANOVA
394 followed by Tukey's *post hoc* test ($p < 0.05$). E, Immunoblot showing the protein level of
395 ABI5 in *ABI5OE* and *bbx30bbx31ABI5OE* upon ABA treatment. In (A), (C) and (E) 1-day-
396 old, germinated seeds were transferred from 0.5x MS -sucrose medium containing plates to
397 similar plates with 10µM ABA for indicated time intervals and then harvested and subjected
398 to immunoblotting using an anti-ABI5 antibody. Actin was used as an internal sample control
399 and blotted using an anti-ACTIN antibody. F, Immunoblot showing the level of ABI5 in

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400 *bbx30 bbx31* and Col-0. Germinated seeds as in (A) were transferred to media containing
401 0.5x MS -sucrose with 10μm ABA or 50μm MG132 for the indicated time. Samples were
402 harvested and blotted using an anti-ABI5 antibody. Actin was used as an internal loading
403 sample control. G, H, Immunoblot showing the protein levels of ABI5 in Col-0, *BBX31OE*,
404 and *bbx30bbx31* upon CHX treatment. Germinated seeds were treated with 20μM ABA for
405 12 hours in the presence of 100μM CHX and samples were harvested at different time
406 intervals. H3 protein was used as a sample loading control. Proteins were detected using
407 Anti-ABI5 and Anti-H3 antibody respectively. In (E-H) numbers below each blot indicate the
408 relative band intensity of proteins that is normalized to the loading control (ACTIN/H3). The
409 intensity of the first band was set to 100.
410
411 **BBX30 and BBX31 enhance ABI5-mediated gene expression by promoting the binding**
412 **of ABI5 on its target promoters**
413
414 We now asked the question if BBX30 and BBX31 can modulate ABI5 activity. ABI5 is
415 known to directly activate its own transcription and also a range of other target genes such as
416 *EM1* and *EM6* (Chen et al., 2008c; Xu et al., 2014; Finkelstein and Lynch, 2000; Leung and
417 Giraudat, 1998). First, we compared the expression level of *ABI5* and *EM6*, in Col-0,
418 *bbx30bbx31, bbx30, bbx31, BBX30OE, BBX31OE* under -ABA and +ABA conditions in
419 tissues of two different developmental stages - in germinated seeds that have completed the
420 radicle emergence and in developing seedlings that have started but not completed the
421 cotyledon emergence. We observed no significant difference in the expression of *ABI5* and
422 *EM6* between the genotypes in the germinated seeds (Figure 7, A and C). However, the
423 emerging seedlings of *BBX30OE* and *BBX31OE* showed higher expression of *ABI5* and *EM6*
424 as compared to Col-0 in the presence of ABA, whereas the expression was lower in the loss
425 of function mutants (Figure 7, B and D). Furthermore, we performed a transient assay in
426 Arabidopsis protoplasts using luciferase gene driven by *ABI5* promoter as the reporter and
427 ABI5, BBX30, and BBX31 as effectors (Figure 7, E). While the three effectors could
428 individually induce the expression of *ABI5pro:LUC*, coexpression of *ABI5, BBX30,* and
429 *BBX31* significantly increased the luciferase activity, suggesting that BBX30 and BBX31
430 enhance the transcriptional activation of target genes by ABI5 (Figure 7, F).
431
432 Subsequently, we performed an electrophoretic mobility shift assay (EMSA) to test the effect
433 of BBX30 and BBX31 on the binding of ABI5 on its target promoters. BBX30 and BBX31
434 did not show direct binding on the promoter regions of *ABI5* and *EM6* where ABI5 is known
435 to bind (Figure 7, G-I) However, the addition of BBX30 and BBX31 along with ABI5
436 resulted in a super shift as compared to ABI5 alone, indicating that BBX30 and BBX31
437 might form a complex with ABI5 during the latter's DNA binding (Figure 7, H and I). To test
438 whether BBX30 and BBX31 influence the binding of ABI5 on its target promoters in vivo,
439 we performed a chromatin immunoprecipitation assay (ChIP) using anti-ABI5 antibody,
440 followed by qPCR using primers flanking the ABI5-binding sites of *EM1* and *EM6* promoters
441 in 3 DAS seedlings of Col-0, *bbx30 bbx31, ABI5OE,* and *bbx30 bbx31 ABI5OE*. The
442 enrichment of ABI5 protein on *EM1* and *EM6* was decreased and increased in *bbx30bbx31*
443 and *ABI5OE*, respectively, as compared to Col-0 (Figure 7, J-L). Interestingly, *bbx30 bbx31*

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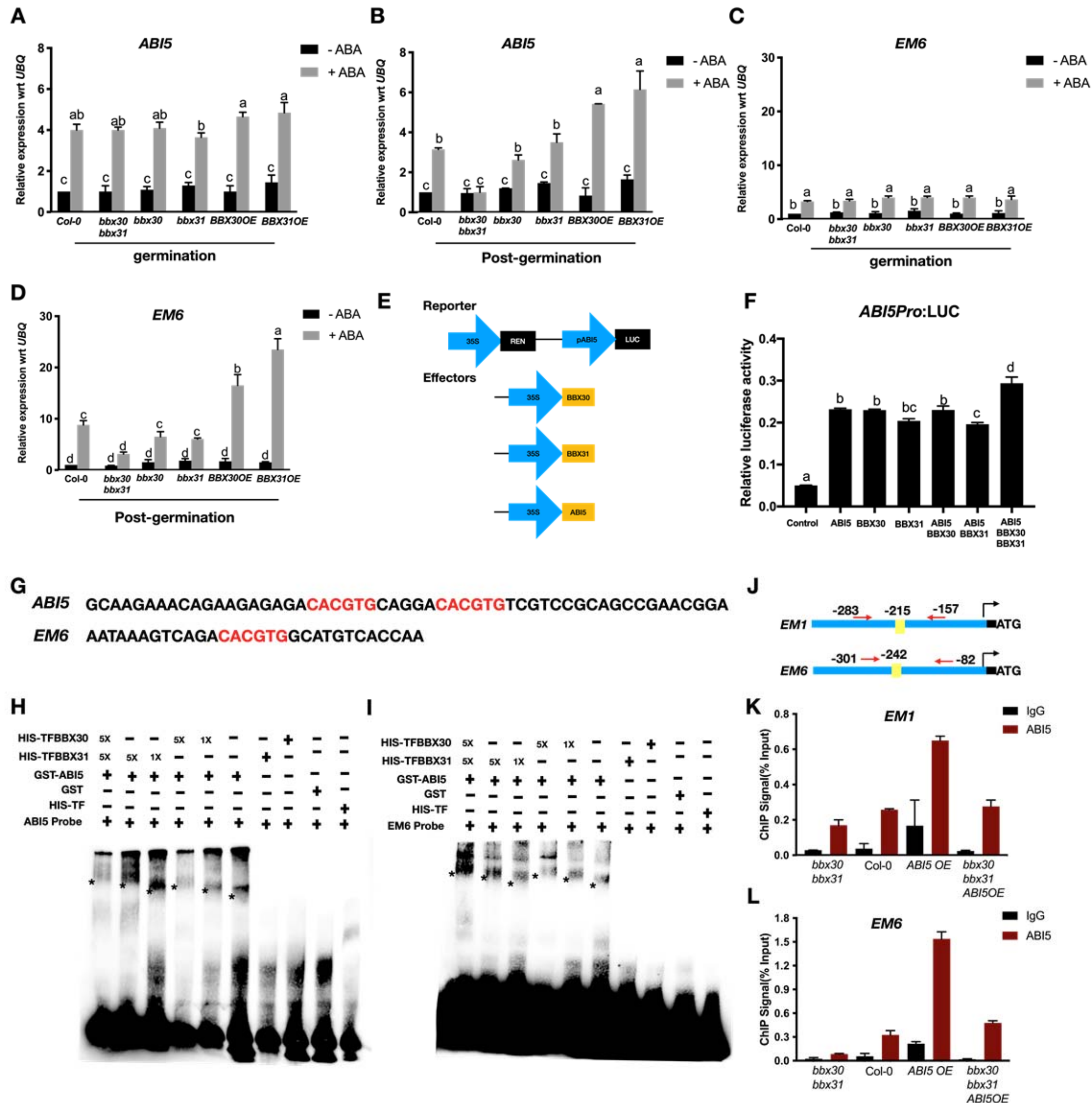
444 *ABI5OE* exhibited less ABI5 protein enrichment on *EM1* and *EM6* as compared to *ABI5OE*,
445 suggesting that binding of ABI5 on the target promoters is promoted by BBX30 and BBX31
446 (Figure 7, J- L).



447

448 **Figure 7. BBX30 and BBX31 enhance ABI5-mediated gene expression by promoting the**
449 **binding of ABI5 on its target promoters**
450 A-D, Relative expression of (A-B) *ABI5* and (C-D) *EM6* in Col-0, *bbx30 bbx31, bbx30,*
451 *bbx31, BBX30 OE, BBX31 OE*. Seeds were inoculated on 0.5x MS -sucrose plates, with or
452 without 1µm ABA. The seeds were harvested on the second day (germination) and the third
453 day (post-germination) after stratification. Values are the mean of three biological replicates.
454 E, Schematic representation of the constructs used in the transient assay in *Arabidopsis*
455 protoplast. The reporter construct used is based on the pGreen vector expressing *35S*
456 *promoter* driven Renilla Luciferase and $ABI5_{pro}$ driven Luciferase. Effectors used are *ABI5,*

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457 *BBX30, BBX31* cloned under the constitutive *35S* promoter. F, Relative luciferase activity,
458 showing the activation of $ABI5_{pro}$: LUC by the combination of proteins mentioned. Error bar
459 represents SD (n=3). G, The sequence of promoter regions of *ABI5* and *EM6* used as probes
460 for the EMSA. The red highlighted region is the G- box type ABRE *cis*-regulatory element.
461 H, I, EMSA showing the binding of ABI5 along with BBX30 and BBX31. BBX30 and
462 BBX31 alone, do not bind to *ABI5* and *EM6* promoters. The addition of BBX30 and BBX31
463 in the presence of ABI5 leads to a super shift (denoted by *) of the DNA-protein complex. +
464 and - denotes the presence and absence of the probe or proteins indicated on the left. 1X and
465 5X represent the concentration of BBX30 and BBX31. J, Schematic representation of *EM1*
466 and *EM6* promoter. Black arrow represents the transcription start site. The yellow boxes
467 indicates G-box type ABRE *cis*-regulatory elements, and the number represents the base
468 location of G-box type elements. Red arrows denote primer binding sites for the primers used
469 to check enrichment by qPCR following ChIP. K, L, ChIP-qPCR analysis determining the
470 binding of ABI5 onto the promoter of (K) *EM1* and (L) *EM6* in seedlings of *bbx30bbx31*,
471 Col-0, *ABI5OE* and *bbx30 bbx31ABI5OE.* The genotypes used were grown in 1um ABA and
472 samples were harvested on the third day after stratification. DNA-Protein complexes were
473 immunoprecipitated using anti-ABI5 and anti-IgG antibodies (negative control). ChIP DNA
474 was quantified using primers flanking the G-box region of *EM1* and *EM6*. Letters above the
475 error bar (A-D and F) indicated the statistical groups as determined by one-way ANOVA
476 followed by Tukey's *post hoc* test ($p < 0.05$).
477

478 **ABI5 directly binds to the promoters of *BBX30* and *BBX31* and induces their**
479 **transcription**
480

481 *BBX30* and *BBX31* are well characterized as light-responsive genes induced during dark to
482 light transition and are also regulated by UV-B, and the circadian clock (Yadav et al., 2019;
483 Wu et al., 2020; Graeff et al., 2016). Since our data suggest the important role of BBX30 and
484 BBX31 in ABA response during post-germination development, we asked whether these
485 genes are transcriptionally regulated by ABA. We examined the expression levels of *BBX30*
486 and *BBX31* by RT-qPCR in germinated Col-0 seeds treated with ABA for 0, 1, 6 and 12
487 hours (Figure 8, A and B)). ABA treatment for 1 hour results in 3-fold and 12-fold
488 upregulation in the mRNA levels of *BBX30* and *BBX31* respectively (Figure 8, A and B).
489 Treatment with ABA for longer durations up to 12 hours results in the expression levels of
490 *BBX30* and *BBX31* elevating to 4-folds and 15-folds compared to no ABA treatment (0 hour)
491 (Figure 8, A and B). The G-box type ABA response elements (ABRE) present in the
492 promoter of *BBX30* and *BBX31* are known to be the target sites of the transcription factor
493 ABI5 that plays crucial roles in ABA signaling. To test if *BBX30* and *BBX31* are induced by
494 ABA in an ABI5-dependent manner, we examined their expression in *abi5-8* treated similarly
495 as explained above for Col-0. We found that the absence of ABI5 impairs the ABA-induced
496 upregulation of *BBX30* and *BBX31* (Figure 8, A and B). All these data suggest that after
497 germination ABA induces the expression of *BBX30* and *BBX31* in a ABI5 dependent manner.
498 Next, we asked the question if ABI5 can directly bind to the promoter of *BBX30* and *BBX31*.
499 To examine this, we performed EMSA using GST-ABI5 protein and biotinylated *BBX30* and
500 *BBX31* promoter regions as the probes. GST-ABI5 was able to bind to the G-box-containing

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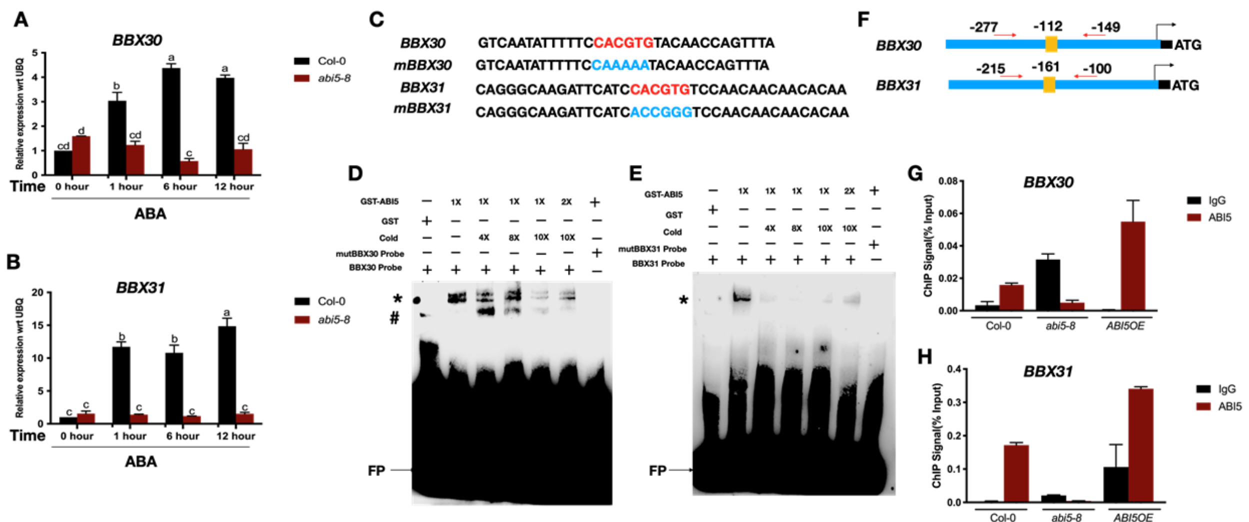
501 promoter sequences of *BBX30* and *BBX31*, while the binding was abrogated in the probes
502 containing mutated G-boxes, indicating that ABI5 directly binds on the G-box elements on
503 the promoters of *BBX30* and *BBX31* (Figure 8, C-E). To validate the in vivo binding of ABI5
504 on these promoters, we also performed a ChIP-qPCR, and examined the regions of *BBX30*
505 and *BBX31* promoters showing ABI5 enrichment. The results indicated that ABI5 indeed
506 binds to *BBX30* and *BBX31* promoters on the regions containing the G-box type ABRE cis-
507 regulatory elements (Figure 8, F-H). These evidences suggest that while BBX30 and BBX31
508 interact with ABI5 to stabilize it and promote the expression of downstream ABA responsive
509 genes, ABI5 establishes a positive feedback loop to directly activate the transcription of
510 *BBX30* and *BBX31* (Figure 9).
511



512
513
514 **Figure 8. ABI5 directly binds to the promoter of *BBX30* and *BBX31* and induces their**
515 **transcription**
516 A-B, The relative expression level of (A) *BBX30* and (B) *BBX31* in the Col-0 and *abi5-8*.
517 Seeds were germinated on 0.5xMS -sucrose conditions. 100% germinated seeds were
518 transferred to medium containing 10µM ABA. Error bars indicate SEM of two biological
519 replicates, the letters above the bars represent different statistical group. C, Sequence of the
520 probes used for EMSA, mutated nucleotides are shown in blue colour. D-E, EMSA showing
521 the binding of ABI5 to the promoter of *BBX30* and *BBX31*, containing the G-box domain and
522 labelled with biotin. *mutBBX30* and *mutBBX31* represent the mutated G-box, while + and -
523 indicate presence and absence; cold represents the unlabelled probes used in 4X, 8X, and
524 10X concentrations. FP represents a free probe. # Denotes the nonspecific shift and *
525 indicates the ABI5 mediated shift. F, Schematic representation of *BBX30* and *BBX31*
526 promoter. Arrow represents the transcription start site. The yellow boxes indicate G-box type
527 ABRE *cis*-regulatory elements, and the number represents the base location of G-box type
528 elements. G-H, ChIP-qPCR analysis determining the binding of ABI5 onto the promoter of
529 *BBX30* and *BBX31* in seedlings of Col-0, *abi5-8,* and *ABI5OE*. The genotypes were grown in
530 1um ABA and samples were harvested on the third day after stratification. DNA-Protein

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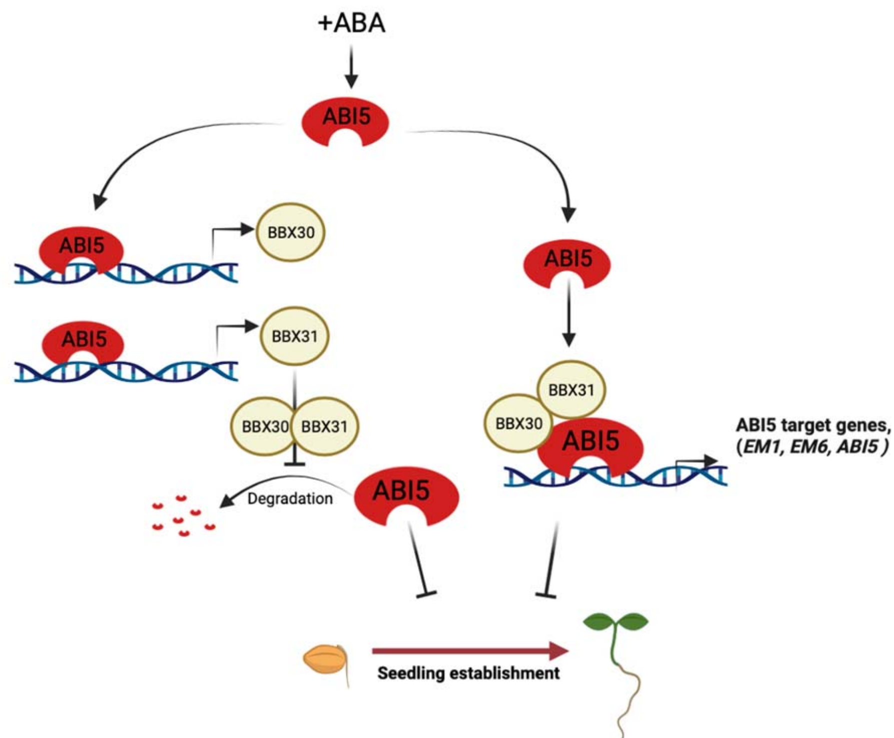
531 complexes were immunoprecipitated using anti-ABI5 and anti-IgG antibodies (negative
532 control). ChIP DNA was quantified using primers flanking the G-box region of *BBX30* and
533 *BBX31*. Error bar represents the the mean ± SEM of three technical replicates.
534
535



536 **Figure 9**. **Model showing BX30, BBX31 and ABI5 regulate post-germination seedling**
537 **development in an interdependent manner.** BBX30 and BBX31 promote the stability of
538 ABA-induced ABI5 by inhibiting its degradation and regulating its activity to promote
539 expression of ABI5 target genes. ABI5 on the other hand directly binds to the promoter of
540 *BBX30* and *BBX31* and promotes their transcription. The microproteins miP1a/BBX31 and
541 miP1b/BBX30 alongwith ABI5 thereby form a positive feedback loop to inhibit seedling
542 establishment.
543

544 **Discussion**

545 Germination, seedling development and stomatal activity are among the several important
546 developmental events regulated by both ABA and light (Yadukrishnan and Datta, 2021; Ali
547 et al., 2020; Xu et al., 2014; Chen et al., 2008; Lau and Deng., 2007). Of these the integrated
548 role of ABA and light in regulating early seedling development is relatively less known.
549 ABA inhibits post-germination seedling establishment and the ABA sensitivity of seedlings
550 is known to be modulated by light. ABI5 seems to play a key role in mediating the interplay
551 between the light and ABA signaling pathways (Xu et al., 2014; Collin et al., 2021). Several

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552 members of the light signaling pathway that are modulated by the light or dark cue, feed the
553 light information either upstream or downstream of ABI5. A number of light signaling
554 factors have been identified to bind to the promoter of *ABI5* and regulate its transcription.
555 These include transcription factors like PIFs, DET1, HY5, BBX19, BBX21, FAR1 and
556 FHY3 (Sakuraba et al., 2014; Penfield et al., 2010; Shi et al., 2013; Xu et al., 2014; Kang et
557 al., 2018; Bai et al., 2019). Some other light signaling regulators can modulate ABI5 activity
558 at the protein level. This may be as a result of direct physical binding with ABI5 as in the
559 case of PIF1 or indirectly modulating ABI5 stability by binding to its repressor ABD1 as
560 mediated by COP1 (Peng et al., 2022). The stabilization activities of PIF1 and COP1 are
561 limited to the dark environment. COP1 also promotes ABI5 mediated downstream gene
562 activation to suppress post-germination seedling establishment (Yadukrishnan et al., 2020).
563 PIF1 and ABI5 act in a cooperative manner to activate common downstream target genes by
564 binding to their promoters in the dark(Kim et al., 2016).To prevent precocious seedling
565 establishment ABI5 needs to be active both under dark and in light. Here we identified two
566 microProteins miP1a/ BBX31 and miP1b/BBX30 that directly interact with ABI5 to stabilize
567 it and promote its downstream activities in the light (Figure 5, 6, 7). ABI5 via a positive
568 feedback loop regulates the transcription of miP1a/BBX31 and miP1b/BBX30 (Figure 8).
569 The microProteins and ABI5 thus mediate seedling growth arrest in an interdependent
570 manner, at least partially (Figure 1, 2, 3, 4). This study provides a novel microprotein
571 mediated post-translational regulatory mechanism for controlling ABI5 accumulation and
572 activity during post-germination seedling establishment in the absence of COP1and PIFs that
573 are deactivated in light.

574 BBX proteins are zinc-finger transcription factors that regulate numerous aspects of early
575 seedling development (Yadav et al., 2020, 2020; Song et al., 2020). In Arabidopsis there are
576 32 B-box proteins that are divided into five structural groups based on the domain structure
577 (Gangappa and Botto, 2014). The structural groups I-III (BBX1-BBX17) contain a CCT
578 domain at the C-terminal half which is often involved in DNA binding and transcriptional
579 regulation. BBX11 regulates the accumulation of protochlorophyllide to optimize greening
580 during de-etiolation (Job, 2020). Structural group IV contains 2 B-box domains but is devoid
581 of the CCT domain. Four members of this group (BBX20, BBX21, BBX22, BBX23) have
582 been characterized as positive regulators of photomorphogenesis while the other four
583 (BBX18, BBX19, BBX24, BBX25) repress photomorphogenic development. BBX20,
584 BBX21, BBX22 act as cofactors of HY5 to mediate light-regulated development (Bursch et
585 al., 2020; Job et al., 2018). An M6 motif identified in the C-terminus of these proteins might
586 regulate their specific developmental responses in response to light (Yadukrishnan et al.,
587 2018). Structural group five consists of 7 members (BBX26-BBX32) that contain only one
588 B-box domain. Functional characterization of these single-domain BBX proteins indicates
589 that many of them play important roles in early seedling development. BBX30 and BBX31
590 promote hypocotyl elongation in seedlings under visible light (Yadav et al., 2019; Heng et
591 al., 2019). HY5 inhibits the transcription of *BBX30* and *BBX31* (Heng et al., 2019).
592 Interestingly, BBX30 and BBX31 positively regulate *BBX28* and *BBX29* which suppresses
593 HY5-mediated inhibition to promote the transcription of *BBX30* and *BBX31* to fine-tune
594 photomorphogenic development (Song et al., 2020). BBX32 interacts with BBX21 and

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595 suppresses the promotion of light-mediated gene expression by BBX21 and HY5 (Holtan et
596 al., 2011). Wu et al elegantly characterized the specific role of BBX30 and BBX31 during
597 de-etiolation (Wu et al., 2020). These two proteins inhibit the dimerization of PIF3 and EIN3
598 to promote apical hook and cotyledon opening during the dark to light transition. It is
599 interesting to note that other group V members BBX32, BBX28 and BBX29 promote BR-
600 mediated cotyledon closure in the dark (Ravindran et al., 2021; Cao et al., 2022). The
601 functional diversity between these closely related BBX proteins needs further
602 characterization. Our study here shows that BBX30 and BBX31 inhibit post-germination
603 seedling establishment in an ABA-dependent manner. It seems that the same proteins may be
604 recruited for different functions depending on the presence or absence of stress conditions. In
605 addition to their role in modulating early seedling development, BBX30 and BBX31 also
606 regulate flowering and BBX31 promotes UV-B stress tolerance (Yadav et al., 2019; Graeff et
607 al., 2016).

608 MicroProteins miP1a/BBX31 and miP1b/BBX30 are 121 and 117 amino acids long
609 respectively and show 65.5% sequence identity (Graeff et al., 2016). microProteins form
610 homodimers, heterodimers, or multimeric complexes by interacting with the protein-protein
611 interaction domains of their targets. As of now, not more than 50 miPs have been functionally
612 characterized in plants. In most of these cases, the microProteins repress the activity of their
613 targets (Staudt and Wenkel, 2011). In fact, miP1a and miP1b have been previously shown to
614 form a repressor complex together with CO and TOPLESS to inhibit flowering (Graeff et al.,
615 2016). This microProtein duo also inhibits the oligomerization of PIF3 and EIN3 to repress
616 their transcriptional activation potential (Wu et al., 2020). Here we report that miP1a/BBX31
617 and miP1b/BBX30 physically interact with ABI5, stabilize it and promote its binding to the
618 downstream targets to activate their expression (Figure 5,6,7). The versatility in the mode of
619 action of the same miPs probably depending on the developmental stage of the plant and the
620 environmental conditions is intriguing. During dark to light transition, miP1a/BBX31 and
621 miP1b/BBX30 promote seedling de-etiolation, however, in the presence of ABA, these miPs
622 arrest seedling development. What is the basis of this differential regulation in the presence
623 or absence of stress is yet to be discovered. Our data shows that ABA induces the expression
624 of *miP1a/BBX31* and *miP1b/BBX30* in a ABI5 dependent manner (Figure 8). ABI5 binds to
625 the G-box motif present in the promoters of *miP1a/BBX31* and *miP1b/BBX30* to activate their
626 expression (Figure 8). Previously HY5 has been shown to bind to the G-box of *BBX30* and
627 *BBX31* to repress their transcription in light(Heng et al., 2019; Yadav et al., 2019). However
628 this differential transcriptional regulation of the miPs is unlikely to account for their varied
629 post-translational activity in presence or absence of ABA. The upregulation of *miP1a/BBX31*
630 and *miP1b/BBX30* expression is extremely rapid and transient happening within the 1 hour of
631 the transfer from dark to light (Wu et al., 2020). The inhibitory activity of miP1a/BBX31 and
632 miP1b/BBX30 in preventing PIF3 and EIN3 functional oligomer formation also seems to be
633 transient as PIF3 and EIN3 are eventually degraded by light. It is possible that the miPs might
634 be disengaged from this transient activity in presence of stress. It would be interesting to see
635 what happens to the allosteric deactivation of PIF3 and EIN3 by miP1a and miP1b in
636 presence of ABA. The cooperative action of miP1a and miP1b in interacting with its targets
637 is also a unique feature of this regulatory module that needs further characterization. In

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638 several eukaryotes, miPs regulate transitions in the cell cycle and in the circadian clock. In
639 yeast, the microProtein Nrs1 rewires the transcriptional machinery to regulate G1/S transition
640 under nutrient stress conditions. This study and some previous reports indicate the role of
641 miP1a/BBX31 and miP1b/BBX30 in modulating the transition from seed to seedling and
642 vegetative to flowering state (Wu et al., 2020; Graeff et al., 2016). In the plant kingdom,
643 miPs are generally present in dicotyledonous flowering plants and might have evolved to
644 modulate these crucial transitions that regulate plant life and productivity (Graeff et al.,
645 2016). The identification of light regulated microProteins in modulating ABA sensitivity
646 during the early establishment phase, might open the doors for optogenetic manipulation of
647 these versatile regulators. In the future, synthetic miPs may be used as molecular brakes to
648 postpone development during unfavourable conditions to ensure plant survival.

649

650 **Materials and methods**

651

652 **Plant materials and growth conditions**

653 In this study, the accession of *Arabidopsis thaliana* used is Columbia-0 (Col-0). The mutant
654 and overexpresser lines *bbx30, bbx31, BBX30OE, BBX31OE, abi5-8, and ABI5OE* have been
655 described previously (Graeff et al., 2016; Yadav et al., 2019; Yadukrishnan et al., 2020;
656 Nambara et al., 1995). The double and triple mutants used in this study were generated by
657 genetic crossing. The growth conditions and ABA treatments were similar to those described
658 previously (Yadukrishnan et al., 2020). In short, the seeds were surface sterilized with
659 sodium hypochlorite and stratified in water for 3 days. The seed was then sown on 0.5x MS (-
660)sucrose plates containing 1% agar and transferred to 16 h/8 h light/dark cycles of 80 µmol
661 m^{-2} sec^{-1} of white light and 22°C in a Percival (CU-41L4) growth chamber for the desired
662 number of days.

663

664 **Plasmid construction**

665 To generate His-BBX30 and His-BBX31 constructs, the full-length coding sequences of
666 *BBX30* and *BBX31* were cloned into the *BamHI-EcoRI* sites of the pET-28a vector and
667 pGEX4T vector respectively. To generate GST-ABI5 construct the full-length *ABI5* was
668 cloned in EcoRI-XhoI sites of the pGEX-4T-1. To generate YFP^c- BBX30 and YFP^c- BBX31
669 construct, the coding sequence of *BBX30* and *BBX31* were amplified, and the cloned in
670 pDONR207. The BP product was then cloned in destination vector pCL113. Similarly to
671 generate YFP^c-ABI5, full-length ABI5 was cloned in pDONR207, and the BP product was
672 cloned in pCL112. To create Yeast two-hybrid plasmid, the full-length coding region of
673 *ABI5, BBX30* and *BBX31* were cloned in pGBKT7 (BD) and pGADT7 (AD) respectively. To
674 study the domain interaction, we cloned different domains of ABI5 in the AD vector and full-
675 length BBX31, N-terminal, and C-terminal of BBX31 in the BD vector. For the luciferase
676 experiment, the reporter construct was generated by amplifying 1kb promoter of the *ABI5*
677 gene followed by cloning at the KpnI-PstI sites of the pGreen II 0800-LUC vector (Lin et al.,
678 2018). The effectors were generated by amplifying the coding sequence of the *BBX30,*
679 *BBX31, and ABI5* gene, followed by its cloning in the pCAMBIA1300 vector using gateway

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680 cloning. All primers used to create the above-mentioned constructs are listed in Supplemental
681 Table S1. The constructs were confirmed via sequencing before use.
682

683 **Quantification of germination and seedling establishment**

684 Seeds of different genotypes were grown and harvested at the same time. The seeds were
685 sterilized and stratified in water for 3 days. The seeds were then inoculated on 0.5x MS plates
686 devoid of sucrose and supplemented with 0μM, 0.5μM, 1μM, 1.5μM of ABA. For estimating
687 % germination, the seeds with completely emerged radicle were counted whereas for %
688 seedling establishment seedlings with open green cotyledons were considered. All
689 germination and seedling establishment experiments were performed thrice with >100 seeds
690 per experiment. All the observations were made and representative images captured using
691 Leica S6E stereomicroscope (Leica Microsystems, www.leica-microsystems.com).
692

693 **Yeast two-hybrid assays**

694 The protein interaction study was performed as described previously (Ravindran et al., 2021).
695 Briefly, the respective combinations of BBX30, BBX31, and ABI5 were co-transformed into
696 the yeast strain *AH109*. Yeast transformants were then grown on the selection medium
697 lacking leucine and tryptophan (DDO/-Trp-Leu). The interaction was further confirmed by
698 growing on a medium lacking Trp, Leu, Ade, and His (QDO/-Trp-Leu-Ade-His).
699

700 ***In-Vitro* pull-down assay**

701 The His-BBX30, His-BBX31, and GST-ABI5 constructs were transformed into
702 ArcticExpress (DE3) cells. For GST-ABI5, the secondary culture of ArcticExpress DE3
703 transformed cells was induced by 0.5mM IPTG at 28° C for 4 hours. The lysate was
704 incubated with GST beads (Glutathione Sepharose 4B, GE Healthcare) for 2 hours at 4° C. In
705 the case of His-BBX30 and His-BBX31, the cell lysate was incubated with Ni-NTA beads
706 (Ni Sepharose 6 Fast flow, GE Healthcare) for 45 mins. The GST and HIS protein-bound
707 beads were washed with 1XPBS+300mM NaCl and 1XPBS+300mM NaCl+20mM imidazole
708 respectively two times. GST-ABI5 was eluted using 30mM glutathione and His-tagged
709 proteins were eluted using 250mM imidazole. The eluted proteins were concentrated, and
710 excessive glutathione and imidazole were removed using MERCK concentrators. The
711 purified 0.5μM GST-ABI5 and GST were allowed to bind to the GST beads for 2h at 4°C.
712 The protein-bound beads were then incubated with purified His-BBX30 and His-BBX31 for
713 2 hours at 4°C. The beads were then washed with PBS twice to remove the unbound protein.
714 The protein bound to the beads were separated using 10% SDS gel, followed by blotting
715 using Anti-His (SAB4301134**,** Sigma) and Anti-GST antibody (ab9085, abcam).
716

717 **Bi-molecular fluorescence complementation assay**

718 BiFC was performed as previously mentioned (Gampala et al., 2007). Briefly, the N-terminal
719 and C-Terminal half of YFP vectors carrying full-length *ABI5, BBX30,* and *BBX31*
720 respectively were transformed into Agrobacterium cells. The bacteria were grown overnight,
721 briefly pelleted and resuspended in infiltration buffer (10mM MES, pH 5.7, 10mM MgCl2,
722 150mM acetosyringone). The suspensions for the constructs as required for the

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723 transformation were mixed and infiltrated into young, fully expanded leaves of *N.*
724 *benthamiana*. The infiltrated leaves were imaged after 2 days using an FV-3000 Olympus
725 confocal microscope with excitation of 488 nm and emission between 510-525 nm.

726

727 **Immunoblotting**

728 For Anti-ABI5 immunoblots, the germinated seeds of Col-0, *bbx30bbx31, BBX31OE,*
729 *ABI5OE,* and *bbx30bbx31ABI5OE* were transferred to media containing 10 μM ABA for
730 indicated time intervals and the harvested samples were crushed in liquid nitrogen and
731 homogenized in extraction buffer (50 mM Tris–HCl, pH 7.5, 75 mM NaCl, 10 mM EDTA,
732 10 mM MgCl2, 0.1% Tween 20, 1mM NaF, and 1 X proteases inhibitor mix, Sigma)
733 followed by centrifugation for 10 min at 11,000 g at 4 °C. Protein concentrations were
734 determined by the Bradford method. 40μg of total protein were loaded per well in SDS-
735 acrylamide/bisacrylamide gel and proteins were electrophoretically transferred to a PVDF
736 membrane (Millipore). For treatment with MG132 and ABA, the germinated seeds of Col-0
737 and *bbx30bbx31* were transferred to media containing 0.5x MS -sucrose with 10μm ABA or
738 50μm MG132 for the indicated time. Samples were harvested and blotted using an anti-ABI5
739 antibody. Actin was used as an internal loading sample control. For cycloheximide (CHX)
740 treatment, germinated seeds were treated with 20μM ABA for 12 hours in the presence of
741 100μM CHX and samples were harvested at different time intervals. H3 protein was used as a
742 sample loading control. Proteins were detected using Anti-ABI5 and Anti-H3 antibody. For
743 immunoblotting, the membrane was blocked for 1 hour in Tris-buffered saline-0.1% Tween
744 20 (TBST) containing 5% BSA. After blocking the proteins were probed with antibodies
745 diluted in TBST overnight at 4˚C. The antibodies used were Anti-ABI5 (ab98831; Abcam,
746 www.abcam.com), Anti-ACTIN (Sigma A0480), Anti-H3 (AS10 710, Agrisera). The
747 membrane was then washed thrice with TBST followed by secondary antibody incubation for
748 1 hour at room temperature. The secondary antibody used was goat-raised horseradish
749 peroxidase-conjugated antirabbit IgG (Sigma) in 1:8000 dilution. To develop the membrane,
750 ECL western blotting substrate (PierceTM), was used. The protein bands were visualized
751 using the ChemiDoc MP imaging system (Bio-Rad). The band intensity was measured using
752 ImageJ software.

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

754 Full-length *BBX30* and *BBX31* cloned in pCold-TF vector was transformed into
755 ArcticExpress DE3 cells. The secondary culture was induced with 0.2mM IPTG at 28˚ C for
756 3 hours, and the cell lysate was incubated with Ni-NTA beads for 1 hour at 4˚C. EMSA was
757 performed as described previously(Job et al., 2018). Briefly, 30-50 base pair oligos were
758 labeled using (Biotin 3′ End DNA Labeling Kit; Pierce), followed by annealing of
759 complementary strands. 1μg of mentioned proteins and 2μg in case of 2X, were incubated
760 with 20 fmol biotinylated probes. The binding was checked by running the incubation
761 mixture on 6% (v/v) native polyacrylamide gels in 0.5× TBE. The DNA-protein complex was
762 blotted to a positively charged nylon membrane and imaged using Chemiluminescent Nucleic
763 Acid Detection Module (Thermo Fisher Scientific). The primers used are listed in
764 Supplemental Table S1.

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765 **Chromatin immunoprecipitation (ChIP)-qPCR**

766 The ChIP assay was performed as described previously (Saleh et al., 2008; Komar et al.,
767 2016; Yadukrishnan et al., 2020). Briefly, approximately 1g of seeds were sterilized and
768 grown on a 0.5xMS-suc plate containing 1µM ABA for 3 days. The harvested tissue was
769 crosslinked by fixing in 1% formaldehyde along with vacuum infiltration. The crosslinking
770 was stopped by adding 0.125M glycine to it. Next, chromatin was isolated using a series of
771 extraction buffers as mentioned in (Saleh et al., 2008), followed by sonication using 4 cycles-
772 30 sec on/30 sec off at 4˚C. 10 percent of the total chromatin sample was stored as input, the
773 rest chromatin sample was incubated with antibody-coated beads at 4˚C. The bead-bound
774 chromatin was then processed for reverse crosslinking using 5M NaCl and 65˚C incubation
775 for 4-6 hours. The beads were then removed from the solution using the magnetic rack, and
776 the DNA was purified using a DNA fragment purification kit. The enrichment was checked
777 through qPCR using primers specific to the promoter/protein binding region. The percentage
778 input methods were used to analyze the enrichment. All the primers used are listed in
779 Supplemental Table S1.

780

781 **Luciferase assay**

782 The protoplast isolation was done as described earlier (Sheen, 2002). pGreen II 0800-LUC
783 vector was used to generate the $ABI5_{Pro}$*: LUC* reporter. *BBX30, BBX31,* and *ABI5* cloned
784 under *35S* promoter were used as effectors. The vectors in different combinations were
785 transfected into the protoplast via PEG solution and incubated overnight to ensure the
786 expression of protein and the activation of the promoter. The luciferase activity was
787 measured using the Promega kit (E1910) and *Renilla luciferase* activity was used as an
788 internal control.

789

790 **RNA isolation and qPCR**

791 RNA was isolated from germinated seeds as previously mentioned (Yadukrishnan et al.,
792 2020). cDNA was prepared using Bio-Rad iSCRIPT cDNA synthesis kit as instructed in the
793 manufacturer's protocol. qPCR was performed in LightCycler®96 (Roche,www.roche.com)
794 machine, and TB Green® Premix EX Taq™ II SYBR Green dye was used (TaKaRa,
795 www.takarabio.com) . For internal control, the reference genes used were UBIQUITIN10 and
796 GAPDH. The qPCR primers used are listed in Supplemental Table S1. The values depicted in
797 the graphs represent the three independent experiments.

798

799 **Statistical analysis**

800 All the statistical analyses were performed using Graph Pad Prism 9.0 and Microsoft Excel.
801 To determine the statistical significance, one-way ANOVA was performed followed by
802 Tukey's *post hoc* test. Other details of the analyses are mentioned in the figure legends.

803

804 **Accession numbers**

805 *BBX30* (AT4G15248), *BBX31* (AT3G21890), *ABI5* (AT2G36270), *EM1* (AT3G51810),
806 *EM6* (AT2G40170).

807

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819

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821

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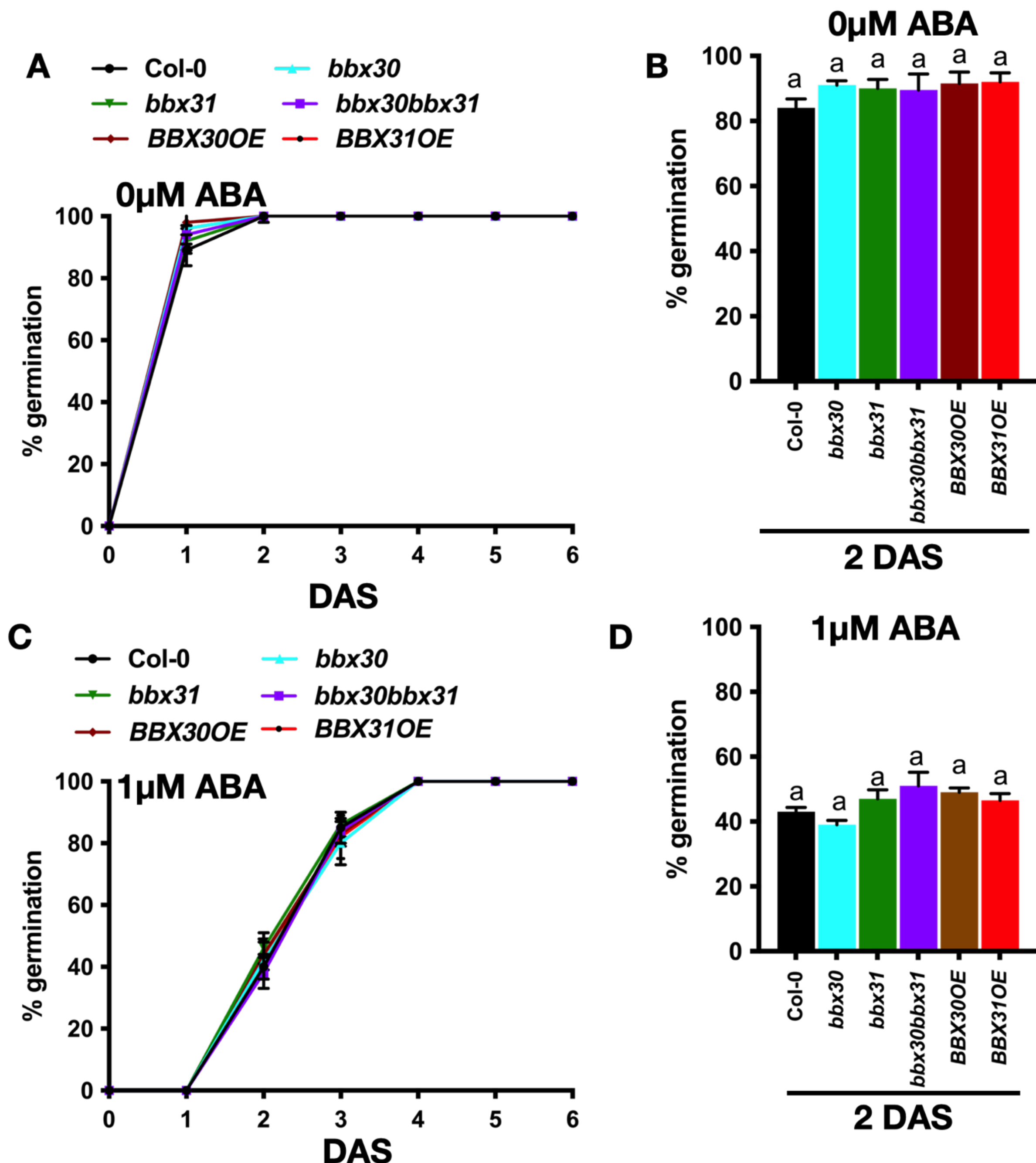
1 **SUPPLEMENTAL DATA**



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4 **Supplemental Figure S1. % germination in Col-0, , *bbx30, bbx31, bbx30 bbx31,***
5 ***BBX30OE and BBX31OE*, in -ABA and +ABA conditions**
6 A-D, Graphical representation showing the similar % germination in the indicated genotypes
7 under (A, B) 0μM and (C, D) 1μM ABA conditions. DAS indicates days after stratification. In
8 (B, D) error bar represents SEM of three independent experiments with >100 seeds per
9 experiment. Letters above the bar indicate the statistical groups as determined by one-way
10 ANOVA followed by Tukey's *post hoc* test ($p < 0.05$).

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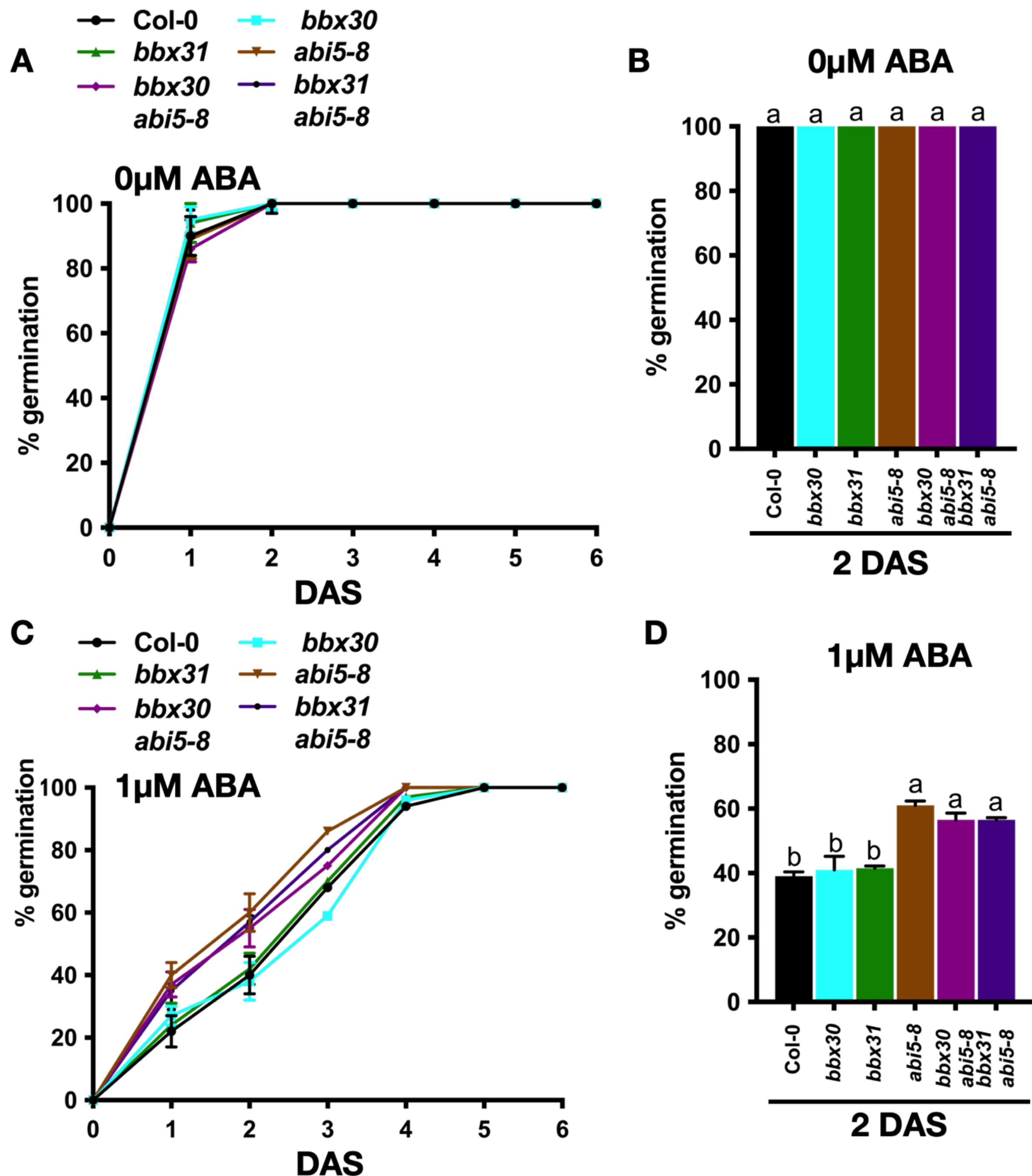

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13 **Supplemental Figure S2. % germination in Col-0*, bbx30, bbx31, abi5-8, bbx30 abi5-8,***

14 ***bbx31 abi5-8*.**

15 A-D, Graphical representation showing the % germination in the indicated genotypes under

16 (A, B) 0µM and (C, D) 1µM ABA conditions. DAS indicates days after stratification. In (B,

17 D) error bar represents SEM of three independent experiments with >100 seeds per experiment.

18 Letters above the bar indicate the statistical groups as determined by one-way ANOVA

19 followed by Tukey's *post hoc* test ($p < 0.05$).

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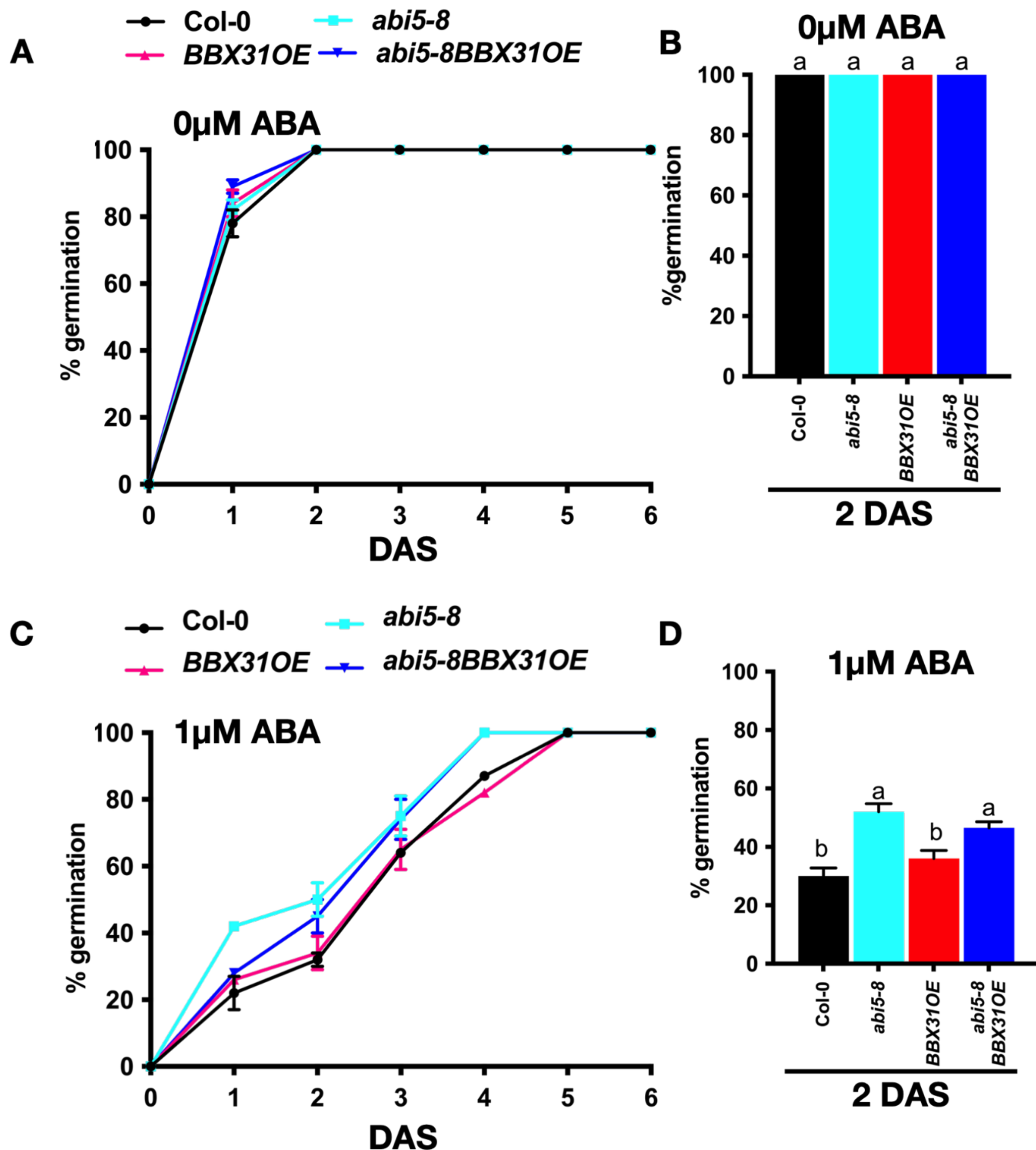

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23 **Supplemental Figure S3. % germination of Col-0, *abi5-8, BBX31OE, abi5-8 BBX31OE***
24 **in +/- ABA conditions**
25 A-D, Graphical representation showing the % germination in the indicated genotypes under
26 (A, B) 0μM and (C, D) 1μM ABA conditions. DAS indicates days after stratification. In (B,
27 D) error bar represents SEM of three independent experiments with >100 seeds per experiment.
28 Letters above the bar indicate the statistical groups as determined by one-way ANOVA
29 followed by Tukey's *post hoc* test ($p < 0.05$).

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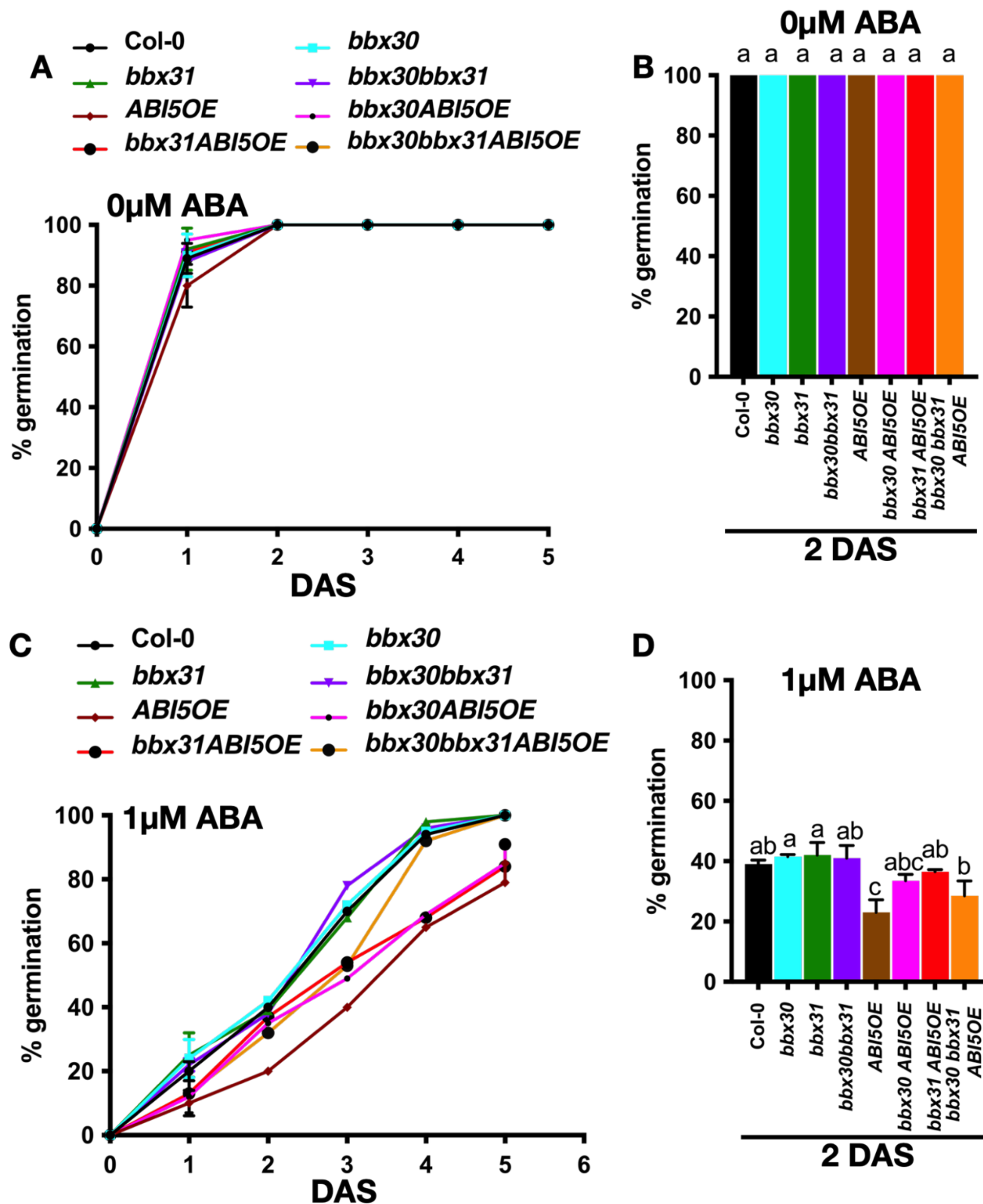

31 **Supplemental Figure S4. % germination of Col-0*, bbx30 bbx31, bbx30, bbx31*, *ABI5OE*,
32 *bbx30 ABI5OE*, *bbx31 ABI5OE*, *bbx30 bbx31 ABI5OE* in +/- ABA conditions**
33 A-D, Graphical representation showing the % germination in the indicated genotypes under
34 (A, B) 0µM and (C, D) 1µM ABA conditions. DAS indicates days after stratification. In (B,
35 D) error bar represents SEM of three independent experiments with >100 seeds per experiment.
36 Letters above the bar indicate the statistical groups as determined by one-way ANOVA
37 followed by Tukey's *post hoc* test ($p < 0.05$).

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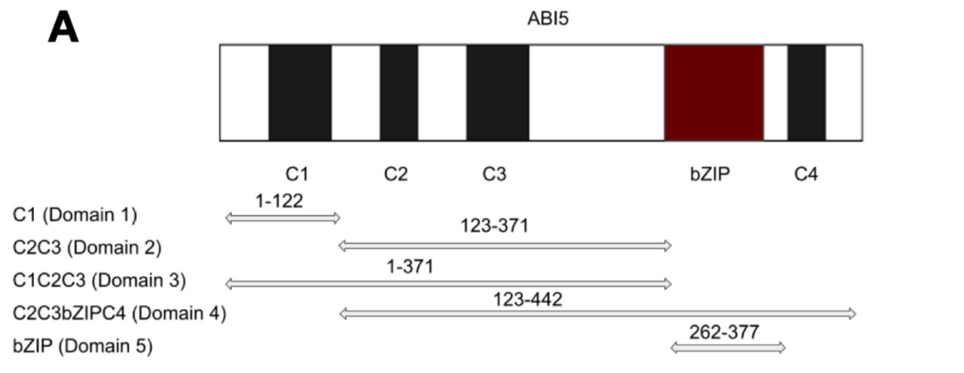

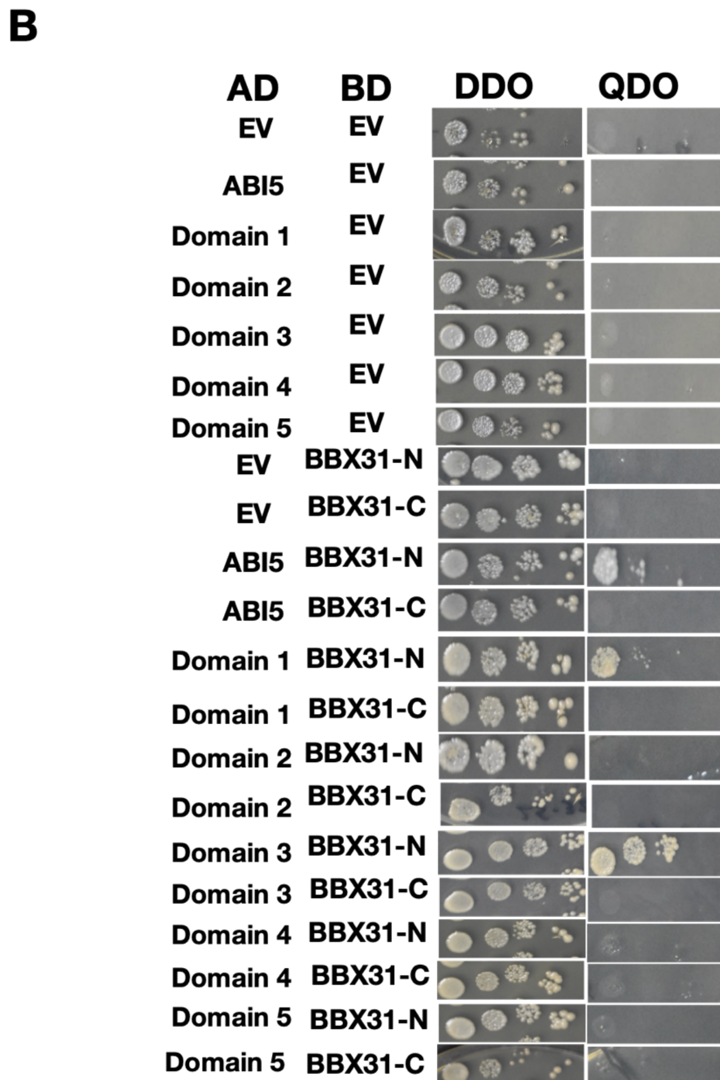

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39 **Supplemental Figure S5. Yeast two hybrid showing interaction between different**
40 **domains of BBX31 and ABI5.**
41 A-B, Yeast two-hybrid assay showing the interaction between the N-terminal and C-terminal
42 domain of BBX31, full length ABI5 and different domains of ABI5. AD and BD represent the
43 GAL4 activation domain and binding domain. N terminal and C-terminal half of BBX31 are
44 fused to AD, while ABI5 and its domains were fused with BD domains and tested for
45 interaction. DDO represents the medium lacking leucine and tryptophan, while QDO
46 additionally lacks histidine and adenine.

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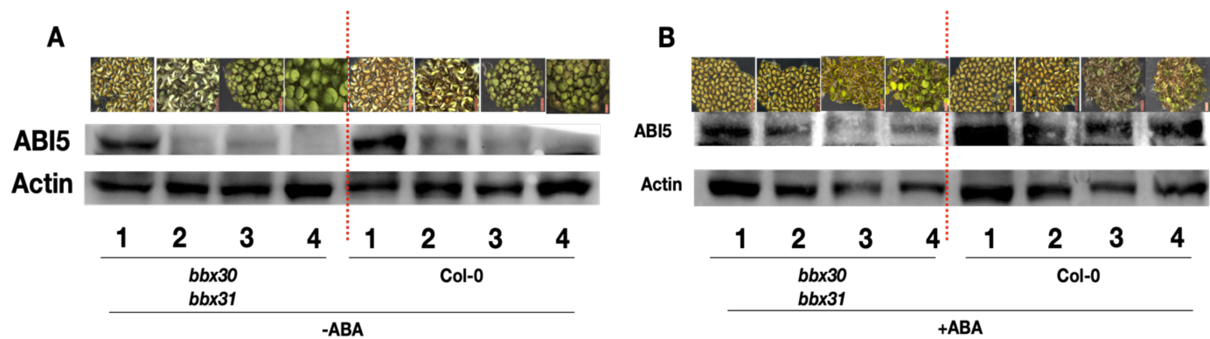

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53 **Supplemental Figure S6. BBX30 and BBX31 promote ABI5 accumulation upon ABA**
54 **treatment**
55 A-B, Immunoblot showing ABI5 accumulation in *bbx30 bbx31* and Col-0 in (A) -ABA and
56 (B) +ABA conditions. Top row shows image of seeds/seedlings inoculated on 0.5x MS-sucrose
57 plates with or without 1μM ABA. Samples were harvested at day 1, 2, 3, and 4 followed by
58 immunoblotting using anti-ABI5 antibody. Actin was used as an internal control.

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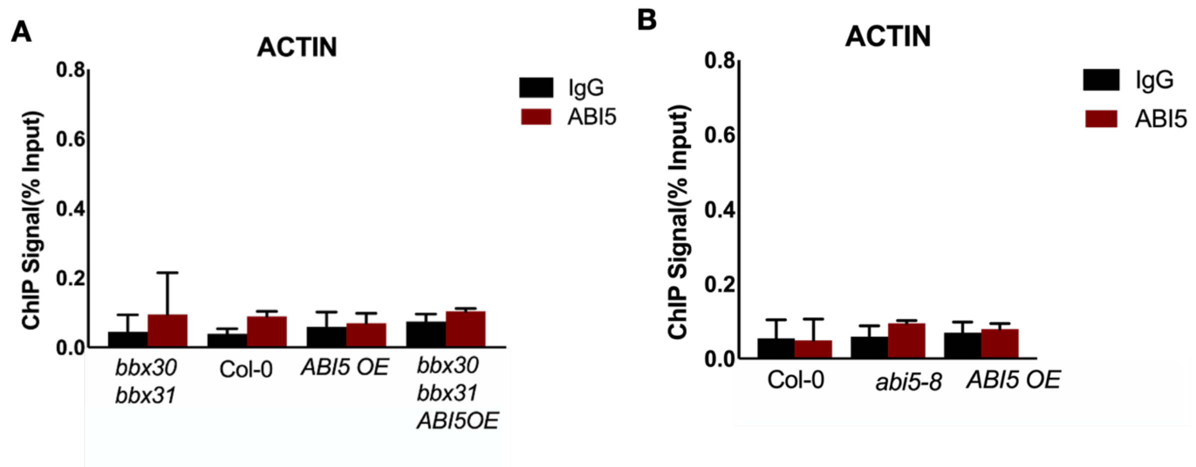

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74 **Supplemental Figure S7. ChIP-qPCR showing the lack of *ACTIN* enrichment over ABI5**
75 A-B, Experimental conditions were same as Figure 7, I and J and Figure 8, G and H. ChIP
76 DNA was quantified using *ACTIN* promoter specific primers.
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102 **Supplemental Table S1. List of primers used in this study**

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Primers for gateway cloning	
BBX30 B1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTGTAGAGGGTTTGAGAAAGAAG
BBX30 B2	GGGGACCACTTTGTACAAGAAAGCTGGGTCGAGAAACACAAAGGGAATTTTGTG
BBX31 B1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGTGTAGAGGCTTGAATAATGA
BBX31 B2	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGAGAAAAACAAACGGAACC
BBX31 NterminalB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTGCGCCTTACGTGTCTCCAAGCT
BBX31 CterminalB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGTGCTATGCACTTCTTGTCAGA
ABI5_Gw_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGTAACTAGAGAAACGAAGT
ABI5_Gw_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTAGAGTGGACAACTCGGG
Primers for genetarting protein constructs	
ABI5 FW	CGCGAATTCATGGTAACTAGAGAAACGAAGTTG
ABI5 RW	CGCCTCGAGTACAAGAAAGCTGGGTGTTAGAG
BBX30 FW	CGCGGATCCATGTGTAGAGGGTTTGAGAAAG
BBX30 RW	CGCCTCGAGTCAGAGAAACACAAAGGGAATTTTG
BBX31 FW	CGCGGATCCATGTGTAGAGGCTTGAATAATGAAG
BBX31 RW	CGCCTCGAGTCAGAGAAAAACAAACGGAACC
Primers for EMSA	
bbx31FW	AAATAACAGGGCAAGATTCATCCACGTGTCCAACAACAACACAACACAAA
bbx31 RW	TTTGTGTTGTGTTGTTGTTGGACACGTGGATGAATCTTGCCCTGTTATTT
bbx31 mutFw	AAATAACAGGGCAAGATTCATCACCGGGTCCAACAACAACACAACACAAA
bbx31 mut Rw	TTTGTGTTGTGTTGTTGTTGGACCCGGTGATGAATCTTGCCCTGTTATTT
BBX30 Fw	GTCAATATTTTTCCACGTGTACAACCAGTTTA
BBX30 Rw	TAAACTGGTTGTACACGTGGAAAAATATTGAC
BBX30 mutFw	GTCAATATTTTTCCAAAAATACAACCAGTTTA
BBX30 mutRw	TAAACTGGTTGTATTTTTGGAAAAATATTGAC
EM6 FW	AATAAAGTCAGACACGTGGCATGTCACCAA
EM6 RW	TTGGTGACATGCCACGTGTCTGACTTTATT
ABI5 FW	GCAAGAAACAGAAGAGAGACACGTGCAGGACACGTGTCGTCCGCAGCCGAACGGA
ABI5 RW	TCCGTTCGGCTGCGGACGACACGTGTCCTGCACGTGTCTCTCTTCTGTTTCTTGC
Primers for ChIP	
BBX30 Fw	GGAGGGAAAATAACTAAATTATTG
BBX30 Rw	CTTGTGTTCAAGATTTAGGTTTA
BBX31 Fw	TGTGCGTCCAATATGAGGTC
BBX31 Rw	TGATGTGTTTGTGTTGTGTTGTT
ACTIN 8 Fw	GCCTCTGTAAATCAAAACCCCA
ACTIN 8 Rv	TCTTTTCGCAGGAACCCAATT
EM1 ChIP Fw	GGATTAAGATTAATCGGAGTCG
EM1 ChIP Rw	GTGGAAGAGAAGACGCGGCGAG
EM6 ChIP Fw	GCGGCGGTATAGTTAAAGAACA
EM6 ChIP Rw	GATGATATACGAAGAAGACT
Primers for genotyping	
LBb1.3	ATTTTGCCGATTTCGGAAC
abi5-8-_LP	CAATGGAAGTTCGGAATCATG
abi5-8 RP	CACTCGTTTTCTTCTTAAAGCG
BBX31- CRISPR Fw	GCAGAAGAAGTGACGGAGGA
BBX31- CRISPR Rv	AGAAAAACAAACGGAACCTCA
BBX31- CRISPR FW	GAAGAAGCGACAATGGAGGATG
BBX31- CRISPR RW	ACGAGTTAGCTTCCGACAGG
Primers for qPCR	
ABI5_QF	GAGAATGCGCAGCTAAAACA
ABI5_QR	GTGGACAACTCGGGTTCCTC
EM1_QF	CGAGCTACTAGTGTCCGCTGCA
EM1_QR	GTAAAACCAACCGGCAACCGCA
EM6_QF	CTTGTCTCCGGTGCTAAG
EM6_QR	CAACAGCATCTCGCTGAAG
UBQ10_QF	GGCCTTGTATAATCCCTGATGAATAAG
UBQ10_QR	AAAGAGATAACAGGAACGGAAACATAGT
BBX30_QF	ATGTGTAGAGGGTTTGAGAAAGA
BBX30_QR	TGCGTCTGCCTCACAATACA
BBX31_QF	ATGTGTAGAGGCTTGAATAATGAAGAG
BBX31_QR	TCACATTTTCTACAGAGGAACGC

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