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

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Abstract:

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

Introduction:

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

Abiotic stress signals induce the accumulation of ABA by elevating its biosynthesis and inhibiting its catabolism. ABA-deficient mutants germinate and establish faster than wildtype, whereas ABA catabolism mutants accumulate more ABA and exhibit longer dormancy periods (Tuteja, 2007; Xiong et al., 2002; Shu et al., 2018). ABA-induced by stress is perceived and channeled through a signaling pathway to induce changes in gene expression that ultimately lead to different stress responses. Studies utilizing the exogenous application of ABA have allowed us to understand better the ABA signaling pathway and ABA-induced responses. The ABA-signaling pathway involves the binding of ABA to PYRABACTIN RESISTANCE1 (PYR)/ PYR1-LIKE (PYL)/ REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) receptors leading to its interaction with type 2C protein phosphatases (PP2Cs). This interaction and retention of PP2Cs by ABA-bound receptors leads to the phosphorylation and release of the kinases SnRK2s, which further phosphorylate downstream transcription factors inducing ABA-

59 mediated gene expression (Ali et al., 2020). Numerous transcription factors in Arabidopsis
60 have been characterized for their roles in ABA signaling and response. The bZIP transcription
61 factor ABSCISIC ACID INSESNITIVE5 (ABI5) was identified in a forward genetic screen for
62 mutants exhibiting ABA insensitivity during germination (Finkelstein and Lynch, 2000).
63 Although ABI5 regulates the ABA-mediated inhibition of germination, it plays a relatively
64 more crucial role in desiccation tolerance and ABA sensitivity during post-germination
65 development (Maia et al., 2014). ABI5 promotes post-germination arrest of seedlings to
66 inhibit their growth and establishment under stress(Lopez-Molina et al., 2001). It binds to the
67 G-box type ABA response elements (ABRE) on the promoters of several target genes including
68 its promoter. EARLY METHIONINE-LABELLED 1 (EM1) and EM6, which code for LATE
69 EMBRYOGENESIS ABUNDANT (LEA) proteins, are some of the key downstream targets of ABI5
70 (Choi et al., 2000; Finkelstein and Lynch, 2000; Carles et al., 2002).
71 Sensitivity to ABA during post-germination seedling development is substantially modulated
72 by light(Yadukrishnan and Datta, 2021). The interactions between the ABA and the light
73 signaling pathway components are reciprocal and multi-layered. ABI5 forms the point of
74 convergence of interactions with light signaling factors like PHYTOCHROME-INTERACTING
75 FACTORs (PIFs), ELONGATED HYPOCOTYL5 (HY5), FAR-RED ELONGATED HYPOCOTYLS3
76 (FHY3), DE-ETIOLATED 1 (DET1), and B-box (BBX) proteins at the transcriptional level
77 (Yadukrishnan and Datta, 2021). In the dark, the PIF proteins – PIF1, PIF3, PIF4, and PIF5 –
78 directly bind to the *ABI5* promoter and induce its transcription(Qi et al., 2020). Additionally,
79 ABI5 physically interacts with PIF1 and enhances the binding of PIF1 onto the promoters of
80 common target genes(Kim et al., 2016). The transcription factor HY5 also directly binds to the
81 promoter of *ABI5* and promotes its expression(Chen et al., 2008a). It was recently identified
82 that HY5 acts downstream of COP1 to negatively regulate ABA-mediated inhibition of
83 postgermination seedling development(Yadukrishnan et al., 2020). FHY3 and DET1 directly
84 bind to the *ABI5* promoter to activate or suppress its expression respectively and thereby
85 optimize seedling greening during early development(Tang et al., 2013). In darkness, DET1
86 stabilizes the PIF proteins to indirectly enhance *ABI5* expression (Dong et al., 2014). BBX
87 proteins are B-box containing zinc finger transcription factors that regulate several aspects of
88 light-mediated development including photomorphogenesis, flowering, shade avoidance,
89 high light, and UV-B tolerance (Gangappa and Botto, 2014; Xu et al., 2014). Some BBX proteins
90 play crucial roles in the light-ABA signaling crosstalk(Vaishak et al., 2019). BBX21 is a

91 transcriptional activator of *HY5* and a positive regulator of photomorphogenesis(Datta et al.,
92 2007; Xu et al., 2014). BBX21 physically interacts with HY5 and inhibits its binding on the *ABI5*
93 promoter(Xu et al., 2014) . BBX21 also interacts with ABI5 and prevents it from binding on its
94 own promoter, thereby reducing *ABI5* expression(Xu et al., 2014). Moreover, BBX21 directly
95 binds to the *ABI5* promoter and recruits a chromatin remodeler protein HRB2
96 (HYPERSENSITIVE TO RED AND BLUE 2) to alter the chromatin structure on the *ABI5* promoter
97 and reduce *ABI5* transcription(Kang et al., 2018). Another B-box protein BBX19 binds to the
98 promoter of *ABI5* and induces its expression to promote ABA-mediated inhibition of
99 germination and seedling development(Bai et al., 2019). CO and COL4/BBX5 also regulate ABA
100 signaling, although the mechanistic details need further investigation (Min et al., 2015)
101 ABI5 function is tightly regulated post-translationally by several factors. The activity of ABI5
102 is turned on by SnRK2s through phosphorylation, while it gets deactivated through
103 dephosphorylation by PP2Cs(Nakashima et al., 2009). Furthermore, its activity is fine-tuned
104 through modifications such as ubiquitination, sumoylation, and S-nitrosylation(Stone et al.,
105 2006; Albertos et al., 2015; Miura and Hasegawa, 2009).The stability and activity of ABI5
106 protein are also modulated by components of the light signaling pathway like COP1. *cop1*
107 shows ABA hyposensitivity during post-germination seedling development(Yadukrishnan et
108 al., 2020). COP1 mediates ABA-induced accumulation of ABI5 by physically interacting with
109 ABA-hypersensitive DCAF1 (ABD1) that targets ABI5 for degradation. COP1 ubiquitinates
110 ABD1 to promote its degradation and thereby enhances ABI5 protein stability in dark(Peng et
111 al., 2022). Additionally, COP1 promotes the binding of ABI5 to its target promoters to inhibit
112 seedling growth (Yadukrishnan et al., 2020). Regulation by microProteins has emerged as a
113 relatively new mechanism of post-translational control of protein abundance and activity in
114 various organisms (Rodrigues et al., 2021; Kruusvee et al., 2022; Kruusvee and Wenkel, 2022;
115 Kushwaha et al., 2022). MicroProteins are small, single-domain proteins generally less than
116 140 amino acids long that share sequence homology with multi-domain proteins (Hong et al.,
117 2005; Dolde et al., 2018). ABI5 inhibits seedling establishment under unfavorable conditions
118 when a seedling tries to emerge into the light from the darkness underneath the soil. The
119 efficacy of this post-germination developmental growth arrest depends on the levels of ABI5
120 (Lopez-Molina et al., 2003). How the stability of ABI5 is regulated, especially under light, and
121 factors modulating its activity is relatively less understood.

122 Here we found that the two light inducible microroteins BBX31/miP1a and BBX30/miP1b (Wu
123 et al., 2020) and ABI5 arrest post-germination seedling development in an interdependent
124 manner. ABI5 directly binds to the promoter of *BBX30* and *BBX31* and induces their
125 transcription. BBX30 and BBX31 physically interact with ABI5 and promote its stabilization.
126 BBX30 and BBX31 also enhance ABI5-mediated gene expression by promoting the binding of
127 ABI5 on its target promoters. Taken together our study suggests that ABI5 and the
128 microProteins BBX30 and BBX31 arrest seedling growth by a positive feedback mechanism.
129

130 **Materials and methods**

131

132 **Plant materials and growth conditions**

133 In this study, the accession of *Arabidopsis thaliana* used is Columbia-0 (Col-0). The mutant
134 and overexpressor lines *bbx30, bbx31, BBX30OE, BBX31OE, abi5-8, and ABI5OE* have been
135 described previously(Graeff et al., 2016; Yadav et al., 2019; Yadukrishnan et al., 2020;
136 Nambara et al., 1995). The double and triple mutants used in this study were generated by
137 genetic crossing. The growth conditions and ABA treatments were similar to those described
138 previously (Yadukrishnan et al., 2020). In short, the seeds were surface sterilized with sodium
139 hypochlorite and stratified in water for 3 days. The seed was then sown on 0.5x MS (-)sucrose
140 plates containing 1% agar and transferred to 16 h/8 h light/dark cycles of 80 µmol m^{-2} sec^{-1} of
141 white light and 22°C in a Percival (CU-41L4) growth chamber for the desired number of days.
142

143 **Plasmid construction**

144 To generate His-BBX30 and His-BBX31 constructs, the full-length coding sequences of *BBX30*
145 and *BBX31* were cloned into the *BamHI-EcoRI* sites of the pET-28a vector and pGEX4T vector
146 respectively. To generate GST-ABI5 construct the full-length *ABI5* was cloned in EcoRI-XhoI
147 sites of the pGEX-4T-1. To generate YFP^c- BBX30 and YFP^c- BBX31 construct, the coding
148 sequence of *BBX30* and *BBX31* were amplified, and the cloned in pDONR207. The BP product
149 was then cloned in destination vector pCL113. Similarly to generate YFP^c-ABI5, full-length
150 ABI5 was cloned in pDONR207, and the BP product was cloned in pCL112. To create Yeast
151 two-hybrid plasmid, the full-length coding region of *ABI5, BBX30* and *BBX31* were cloned in
152 pGBKT7 (BD) and pGADT7 (AD) respectively. To study the domain interaction, we cloned
153 different domains of ABI5 in the AD vector and full-length BBX31, N-terminal, and C-terminal

154 of BBX31 in the BD vector. For the luciferase experiment, the reporter construct was
155 generated by amplifying 1kb promoter of the *ABI5* gene followed by cloning at the KpnI-PstI
156 sites of the pGreen II 0800-LUC vector(Lin et al., 2018). The effectors were generated by
157 amplifying the coding sequence of the *BBX30, BBX31, and ABI5* gene, followed by its cloning
158 in the pCAMBIA1300 vector using gateway cloning. All primers used to create the above-
159 mentioned constructs are listed in Supplemental Table S1. The constructs were confirmed via
160 sequencing before use.
161
162 **Quantification of germination and seedling establishment**
163 Seeds of different genotypes were grown and harvested at the same time. The seeds were
164 sterilized and stratified in water for 3 days. The seeds were then inoculated on 0.5x MS plates
165 devoid of sucrose and supplemented with 0µM, 0.5µM, 1µM, 1.5µM of ABA. For estimating
166 % germination, the seeds with completely emerged radicle were counted whereas for %
167 seedling establishment seedlings with open green cotyledons were considered. All
168 germination and seedling establishment experiments were performed thrice with >100 seeds
169 per experiment. All the observations were made and representative images captured using
170 Leica S6E stereomicroscope (Leica Microsystems, www.leica-microsystems.com).
171
172 **Yeast two-hybrid assays**
173 The protein interaction study was performed as described previously (Ravindran et al., 2021).
174 Briefly, the respective combinations of BBX30, BBX31, and ABI5 were co-transformed into the
175 yeast strain *AH109.* Yeast transformants were then grown on the selection medium lacking
176 leucine and tryptophan (DDO/-Trp-Leu). The interaction was further confirmed by growing on
177 a medium lacking Trp, Leu, Ade, and His (QDO/-Trp-Leu-Ade-His).
178
179 ***In-Vitro* pull-down assay**
180 The His-BBX30, His-BBX31, and GST-ABI5 constructs were transformed into ArcticExpress
181 (DE3) cells. For GST-ABI5, the secondary culture of ArcticExpress DE3 transformed cells was
182 induced by 0.5mM IPTG at 28˚ C for 4 hours. The lysate was incubated with GST beads
183 (Glutathione Sepharose 4B, GE Healthcare) for 2 hours at 4˚ C. In the case of His-BBX30 and
184 His-BBX31, the cell lysate was incubated with Ni-NTA beads (Ni Sepharose 6 Fast flow, GE
185 Healthcare) for 45 mins. The GST and HIS protein-bound beads were washed with

186 1XPBS+300mM NaCl and 1XPBS+300mM NaCl+20mM imidazole respectively two times. GST-
187 ABI5 was eluted using 30mM glutathione and His-tagged proteins were eluted using 250mM
188 imidazole. The eluted proteins were concentrated, and excessive glutathione and imidazole
189 were removed using MERCK concentrators. The purified 0.5µM GST-ABI5 and GST were
190 allowed to bind to the GST beads for 2h at 4˚C. The protein-bound beads were then incubated
191 with purified His-BBX30 and His-BBX31 for 2 hours at 4˚C. The beads were then washed with
192 PBS twice to remove the unbound protein. For other combination 0.5 µM of His-BBX30 and
193 His-BBX31 were bound to the beads and used as bait, while prey proteins GST, and GST-ABI5
194 were allowed to bind to the bead bound bait proteins. The protein bound to the beads were
195 separated using 10% SDS gel, followed by blotting using Anti-His (SAB4301134**,** Sigma) and
196 Anti-GST antibody (ab9085, abcam).
197
198 **Bi-molecular fluorescence complementation assay**

199 BiFC was performed as previously mentioned (Gampala et al., 2007). Briefly, the N-terminal
200 and C-Terminal half of YFP vectors carrying full-length *ABI5, BBX30,* and *BBX31* respectively
201 were transformed into Agrobacterium cells. The bacteria were grown overnight, briefly
202 pelleted and resuspended in infiltration buffer (10mM MES, pH 5.7, 10mM MgCl2, 150mM
203 acetosyringone). The suspensions for the constructs as required for the transformation were
204 mixed and infiltrated into young, fully expanded leaves of *N. benthamiana*. The infiltrated
205 leaves were imaged after 2 days using an FV-3000 Olympus confocal microscope with
206 excitation of 488 nm and emission between 510-525 nm.

207

208 **Protein Co-localisation**

209 Destination vector pGWB661 was used to generate BBX30-RFP and BBX31-RFP, while ABI5
210 was cloned in PGWB6 to generate GFP-ABI5. Three weeks old N. benthamiana were infiltrated
211 with Agrobacterium cells transformed with BBX30-RFP and GFP-ABI5 or BBX31-RFP and GFP-
212 ABI5. After 2days incubation in dark, the tagged proteins were visualised using an olympus
213 live cell microscope. The excitation wavelengths of 488 nm and 561 nm was used for GFP and
214 RFP, respectively.

215

216 **Immunoblotting**

217 For Anti-ABI5 immunoblots, the germinated seeds of Col-0, *bbx30bbx31, BBX31OE, ABI5OE,*
218 and *bbx30bbx31ABI5OE* were transferred to media containing 10 µM ABA for indicated time
219 intervals and the harvested samples were crushed in liquid nitrogen and homogenized in
220 extraction buffer (50 mM Tris–HCl, pH 7.5, 75 mM NaCl, 10 mM EDTA, 10 mM MgCl2, 0.1%
221 Tween 20, 1mM NaF, and 1 X proteases inhibitor mix, Sigma) followed by centrifugation for
222 10 min at 11,000 g at 4 °C. Protein concentrations were determined by the Bradford method.
223 40µg of total protein were loaded per well in SDS-acrylamide/bisacrylamide gel and proteins
224 were electrophoretically transferred to a PVDF membrane (Millipore). For treatment with
225 MG132 and ABA, the germinated seeds of Col-0 and *bbx30bbx31* were transferred to media
226 containing 0.5x MS -sucrose with 10µm ABA or 50µm MG132 for the indicated time. Samples
227 were harvested and blotted using an anti-ABI5 antibody. Actin was used as an internal loading
228 sample control. For cycloheximide (CHX) treatment, germinated seeds were treated with
229 20µM ABA for 12 hours in the presence of 100µM CHX and samples were harvested at
230 different time intervals. H3 protein was used as a sample loading control. Proteins were
231 detected using Anti-ABI5 and Anti-H3 antibody. For immunoblotting, the membrane was
232 blocked for 1 hour in Tris-buffered saline-0.1% Tween 20 (TBST) containing 5% BSA. After
233 blocking the proteins were probed with antibodies diluted in TBST overnight at 4˚C. The
234 antibodies used were Anti-ABI5 (ab98831; Abcam, www.abcam.com), Anti-ACTIN (Sigma
235 A0480), Anti-H3 (AS10 710, Agrisera). The membrane was then washed thrice with TBST
236 followed by secondary antibody incubation for 1 hour at room temperature. The secondary
237 antibody used was goat-raised horseradish peroxidase-conjugated antirabbit IgG (Sigma) in
238 1:8000 dilution. To develop the membrane, ECL western blotting substrate (PierceTM), was
239 used. The protein bands were visualized using the ChemiDoc MP imaging system (Bio-Rad).
240 The band intensity was measured using ImageJ software.

241

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

243 Full-length *BBX30* and *BBX31* cloned in pCold-TF vector was transformed into ArcticExpress
244 DE3 cells. The secondary culture was induced with 0.2mM IPTG at 28˚ C for 3 hours, and the

245 cell lysate was incubated with Ni-NTA beads for 1 hour at 4˚C. EMSA was performed as
246 described previously(Job et al., 2018). Briefly, 30-50 base pair oligos were labeled using (Biotin
247 3' End DNA Labeling Kit; Pierce), followed by annealing of complementary strands. 1µg of
248 mentioned proteins and 2µg in case of 2X, were incubated with 20 fmol biotinylated probes.
249 The binding was checked by running the incubation mixture on 6% (v/v) native polyacrylamide
250 gels in 0.5× TBE. The DNA-protein complex was blotted to a positively charged nylon
251 membrane and imaged using Chemiluminescent Nucleic Acid Detection Module (Thermo
252 Fisher Scientific). The primers used are listed in Supplemental Table S1.

253

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

255 The ChIP assay was performed as described previously (Saleh et al., 2008; Komar et al., 2016;
256 Yadukrishnan et al., 2020). Briefly, approximately 1g of seeds were sterilized and grown on a
257 0.5xMS-suc plate containing 1µM ABA for 3 days. The harvested tissue was crosslinked by
258 fixing in 1% formaldehyde along with vacuum infiltration. The crosslinking was stopped by
259 adding 0.125M glycine to it. Next, chromatin was isolated using a series of extraction buffers
260 as mentioned in (Saleh et al., 2008), followed by sonication using 4 cycles- 30 sec on/30 sec
261 off at 4˚C. 10 percent of the total chromatin sample was stored as input, the rest chromatin
262 sample was incubated with antibody-coated beads at 4˚C. The bead-bound chromatin was
263 then processed for reverse crosslinking using 5M NaCl and 65˚C incubation for 4-6 hours. The
264 beads were then removed from the solution using the magnetic rack, and the DNA was
265 purified using a DNA fragment purification kit. The enrichment was checked through qPCR
266 using primers specific to the promoter/protein binding region. The percentage input methods
267 were used to analyze the enrichment. All the primers used are listed in Supplemental Table
268 S1.

269

270 **Luciferase assay**

271 The protoplast isolation was done as described earlier (Sheen, 2002). pGreen II 0800-LUC
272 vector was used to generate the *ABI5$_{Pro}$: LUC* reporter. *BBX30, BBX31,* and *ABI5* cloned under
273 *35S* promoter were used as effectors. The vectors in different combinations were transfected
274 into the protoplast via PEG solution and incubated overnight to ensure the expression of

275 protein and the activation of the promoter. The luciferase activity was measured using the
276 Promega kit (E1910) and *Renilla luciferase* activity was used as an internal control.
277
278 **RNA isolation and qPCR**
279 RNA was isolated from germinated seeds as previously mentioned (Yadukrishnan et al., 2020).
280 cDNA was prepared using Bio-Rad iSCRIPT cDNA synthesis kit as instructed in the
281 manufacturer's protocol. qPCR was performed in LightCycler®96 (Roche,www.roche.com)
282 machine, and TB Green® Premix EX Taq™ II SYBR Green dye was used (TaKaRa,
283 www.takarabio.com) . For internal control, the reference genes used were UBIQUITIN10 and
284 GAPDH. The qPCR primers used are listed in Supplemental Table S1. The values depicted in
285 the graphs represent the three independent experiments.
286
287 **Statistical analysis**
288 All the statistical analyses were performed using Graph Pad Prism 9.0 and Microsoft Excel. To
289 determine the statistical significance, one-way ANOVA was performed followed by Tukey's
290 *post hoc* test. Other details of the analyses are mentioned in the Fig. legends.
291
292 **Accession numbers**
293 *BBX30* (AT4G15248), *BBX31* (AT3G21890), *ABI5* (AT2G36270), *EM1* (AT3G51810), *EM6*
294 (AT2G40170).
295
296 **Results**
297 **BBX30 and BBX31 promote ABA-mediated inhibition of post-germination seedling**
298 **development**
299 *BBX30* and *BBX31* are well-characterized light-responsive genes playing roles in
300 photomorphogenesis, UV-B signaling, and flowering (Heng et al., 2019; Yadav et al., 2019;
301 Graeff et al., 2016). In one of our previous studies, we found that the expression of several
302 genes involved in ABA response and desiccation tolerance was upregulated in *35S:BBX31*
303 (Yadav et al., 2019). In another study, similar genes were downregulated in *bbx30bbx31*(Heng
304 et al., 2019). *BBBX30* and *BBX31* expression is also upregulated by ABA(Xu et al., 2019). The
305 promoter sequences of *BBX30* and *BBX31* contain Abscisic Acid (ABA) response elements
306 (ABRE). All of these prompted us to ask the question if these genes play a role in ABA-

307 regulated developmental responses. We started studying early seedling development,
308 considering the broad function of ABA in regulating germination and post-germination
309 events. We grew imbibed seeds of Col-0, *bbx30*, *bbx31*, *bbx30bbx31*, *BBX30OE*, and *BBX31OE*
310 on a half-strength MS plate, in the presence (+) or absence (-) of ABA. Seedlings with open,
311 expanded green cotyledons were used to calculate % seedling establishment (Fig. S1a-b). In
312 (-) ABA conditions we did not find significant difference in the % seedling establishment of
313 Col-0, *bbx30*, *bbx31*, *bbx30bbx31*, *BBX30OE*, and *BBX31OE*. Seedlings of all the genotypes
314 showed 100% seedling establishment by 3rd day after stratification (DAS) (Fig. 1a-b,d).
315 However, the % seedling establishment in +ABA conditions was higher in *bbx30, bbx31* and
316 *bbx30bbx31* and substantially lower in *BBX30OE* and *BBX31OE* compared to Col-0 (Fig. 1a, c
317 and e). In order to verify if the difference in seedling establishment rate is due to differences
318 in germination, we determind the % germination by counting seeds with emerged radicle in
319 (-) and (+) ABA conditions. However we did not find difference in % germination among the
320 genotypes in either (-) or (+) ABA containing plates. Seedlings of all the genotypes exhibit
321 100% germination by the 2nd day after stratification (DAS) in -ABA conditions while total
322 germination is achieved at 4 DAS under +ABA conditions (Fig. 1f-i). To further confirm the
323 role of BBX30 and BBX31 in ABA-mediated inhibition of seedling development post
324 germination, we germinated seeds on -ABA plates and then transferred them to +ABA plates
325 to determine seedling establishment rate. About 40% of Col-0 seedlings were able to establish
326 in +ABA conditions (Fig. S2a-c). *bbx30, bbx31, bbx30bbx31* seedlings showed higher seedling
327 establishment compared to Col-0, while the seedling establishment % was only 20% for
328 *BBX30OE* and *BBX31OE* seedlings (Fig. S2a-c). This suggests that BBX30 and BBX31 promote
329 ABA-mediated inhibition of seedling development at the post-germination stage.
330
331

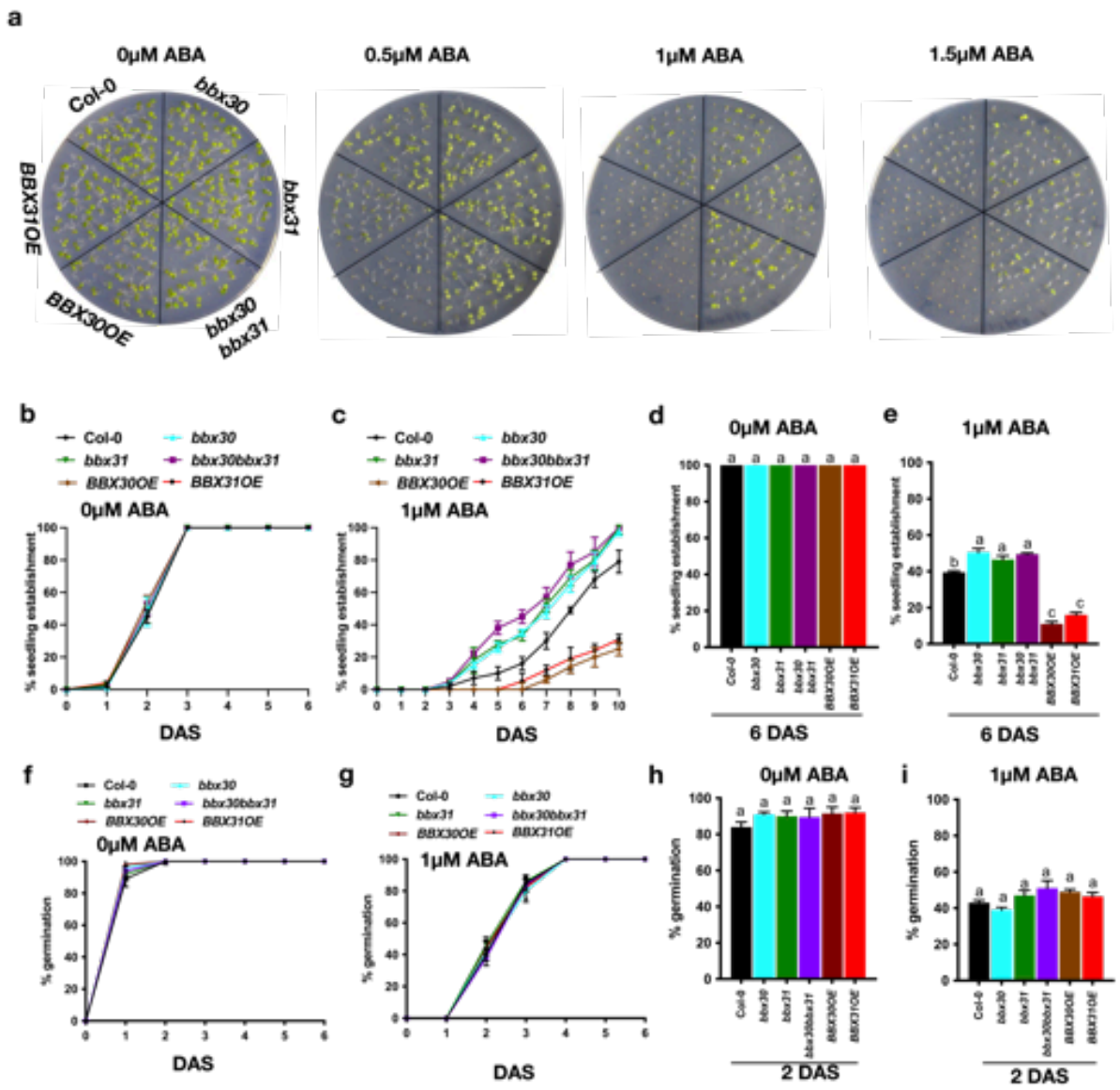
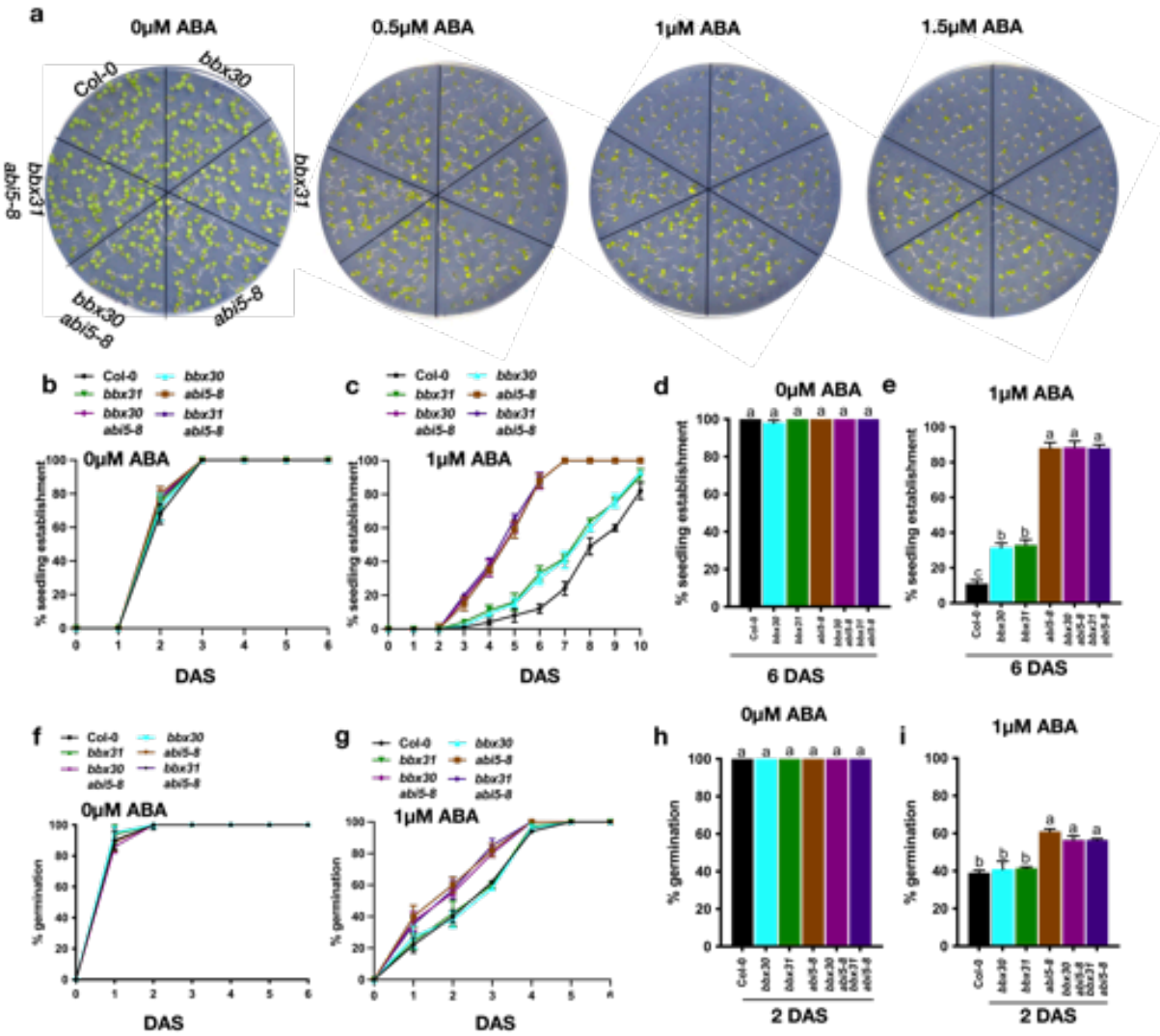


Fig. 1 BBX30 and BBX31 promote ABA-mediated inhibition of post-germination seedling development (a) Representative images of 6-day-old seedlings of Col-0*, bbx30, bbx31*, *bbx30bbx31*, *BBX30OE* and *BBX31OE* grown on a 0.5x MS plate supplemented with 0µM, 0.5µM, 1µM, 1.5µM of ABA. (b-c) % seedling establishment in (b) 0µM ABA and (c) 1µM ABA conditions for the indicated genotypes for up to 10 days after stratification (DAS) and on day 6 (d-e). (f-g) % germination of the indicated genotypes under 0µM (f) and 1µm (g) ABA conditions for up to 6 days after stratification (DAS) and on day 2 (h-i). Seedlings with green, open and expanded cotyledons were counted to determine % seedling establishment. In (b-i) Error bars represent SEM of three independent experiments with >500 seeds. In (d,e,h,i) the letters above the bar indicate the statistical groups as determined by one-way ANOVA followed by Tukey's *post hoc* test ($p < 0.05$).

344 **BBX30, BBX31 and ABI5 mediate post-germination seedling growth arrest in an inter**
345 **dependent manner**
346 The transcription factor ABI5 is a key regulator of several ABA responses including post-
347 germination development and is known to mediate interplay with light signaling factors (Chen
348 et al., 2008b; Yadukrishnan et al., 2020; Lopez-Molina et al., 2001). We asked the question if
349 ABI5 plays any role in BBX30 and BBX31 mediated seedling growth arrest. In the presence of
350 ABA, the percentage of seedling establishment is higher in *abi5-8* compared to Col-0, *bbx30*
351 and *bbx31* (Fig. 2a, c and e). This difference was not seen in -ABA conditions (Fig. 2a, b and
352 d). In order to examine the genetic interaction between *BBX30*, *BBX31* and *ABI5* we generated
353 *bbx30abi5-8* and *bbx31abi5-8* by crossing and monitored the seedling establishment rates of
354 the double mutants under ABA. The establishment rates in *bbx30abi5-8,* and *bbx31abi5-8*
355 were similar to *abi5-8* suggesting that BBX30 and BBX31 regulate these processes in an ABI5-
356 dependent fashion (Fig. 2a-e). There was no difference in % germination among these
357 genotypes in (-) ABA conditions, with all genotypes attaining 100% germination by 2 DAS (Fig.
358 2f and h). In (+) ABA conditions at 2 DAS % germination was higher in *abi5-8, bbx30abi5-8*
359 and *bbx31abi5-8* compared to Col-0, *bbx30* and *bbx31*, however by 5 DAS there was no
360 significant difference in the germination % between the genotypes (Fig. 2g and i).

361

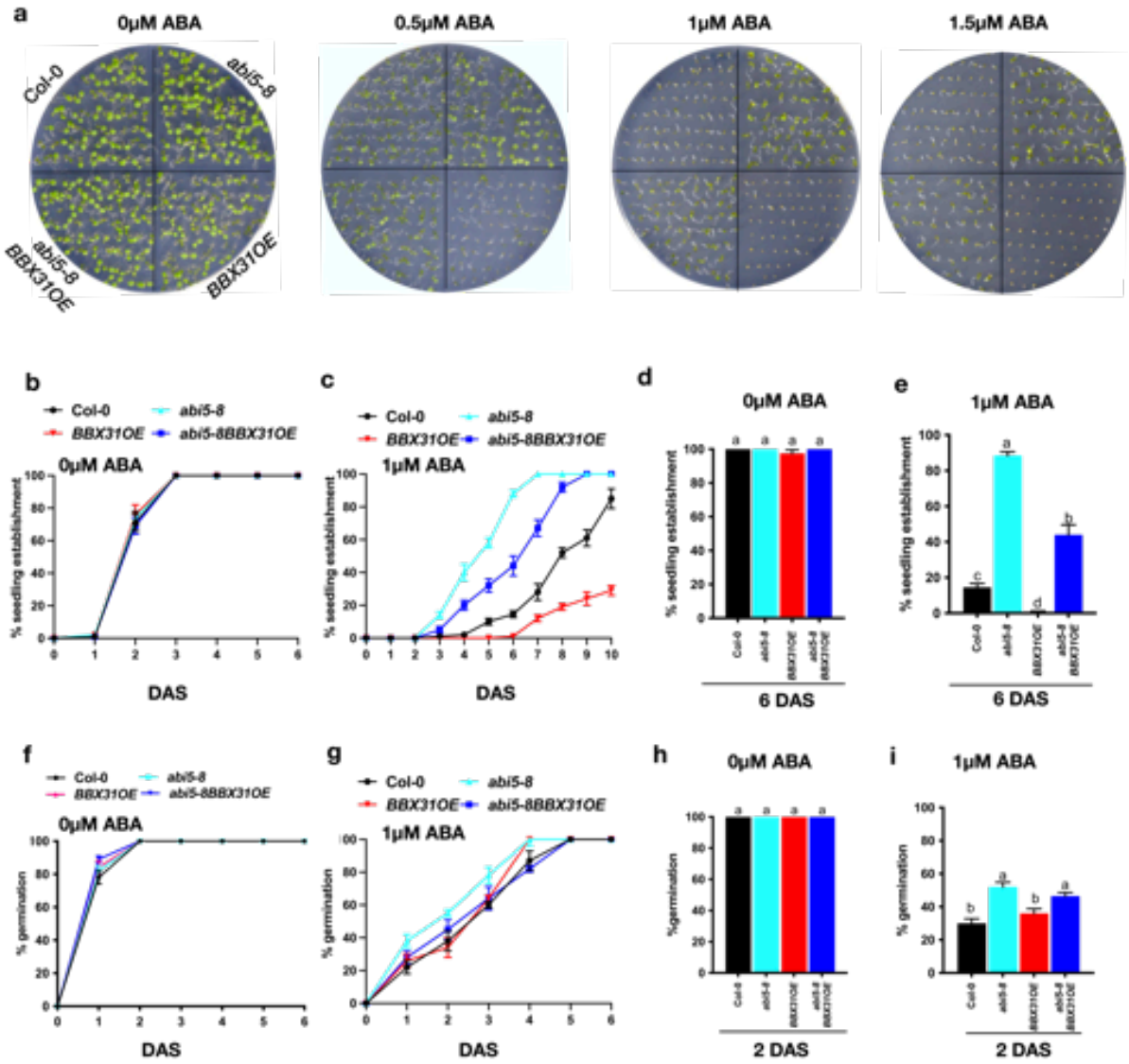


362

363 **Fig. 2 *BBX30, BBX31* and *ABI5* interact genetically to regulate early development in**
364 **Arabidopsis**

365 (a) Representative images of 6 day-old seedlings of Col-0*, bbx30, bbx31*, *abi5-8*, *abi5-8bbx30*,
366 *abi5-8bbx31* grown on 0.5x MS plate supplemented with 0µM, 0.5µM, 1µM, 1.5µM of ABA.
367 (b-c) % seedling establishment in (b) 0µM ABA and (c) 1µM ABA conditions for the indicated
368 genotypes for up to 10 days after stratification (DAS) and on day 6 (d-e). (f-g) % germination
369 of the indicated genotypes under 0µM (f) and 1µM (g) ABA conditions for upto 6 days after
370 stratification (DAS) and on day 2 (h-i). Seedlings with green, open and expanded cotyledons
371 were counted to determine % seedling establishment. In (b-i) Error bars represent SEM of
372 three independet experiments with > 500 seeds. In (d,e,h,i) the letters above the bar indicate

373 the statistical groups as determined by one-way ANOVA followed by Tukey's *post hoc* test (p
374 < 0.05).
375 We further crossed *BBX31OE* with *abi5-8* to obtain *abi5-8BBX31OE* and studied their ABA
376 sensitivity during early seedling development (Fig. 3a). At 6 DAS under 1µM ABA, *abi5-8* and
377 *BBX31OE* exhibit 85% and 2% seedling establishment respectively as compared to 15% in Col-
378 0 (Fig. 3c-e). Seedling establishment % in *abi5-8BBX31OE* is around 40% (Fig. 3, c and e).
379 There is no difference in the % seedling establishment between the genotypes under 0µM
380 ABA (Fig. 3b-d). Germination rates of Col-0, *abi5-8, BBX31OE* and *abi5-8BBX31OE* by 5 DAS
381 in presence or absence of ABA also did not show any significant difference (Fig. 3f-i).These
382 data further validate that BBX31-mediated inhibition of post-germination seedling
383 development is at least partially ABI5 dependent.
384



385 **Fig. 3 BBX31 regulates seedling growth arrest in an ABI5-dependent fashion**
386 (a) Representative images of 6-day-old seedlings of Col-0*, abi5-8, BBX31OE, abi5-8BBX31OE*
387 grown on a 0.5x MS plate supplemented with 0µM, 0.5µM, 1µM, 1.5µM of ABA. (b-c) %
388 seedling establishment in (b) 0µM ABA and (c) 1µM ABA conditions for the indicated
389 genotypes for up to 10 days after stratification (DAS) and on day 6 (d-e). (f-g) % germination
390 of the indicated genotypes under 0µM (f) and 1µM (g) ABA conditions for upto 6 days after
391 stratification (DAS) and on day 2 (h-i). Seedlings with green, open and expanded cotyledons
392 were counted to determine % seedling establishment. In (b-i) Error bars represent SEM of
393 three independent experiments with >500 seeds. In (d,e,h,i) the letters above the bar indicate
394 the statistical groups as determined by one-way ANOVA followed by Tukey's *post hoc* test (p
395 < 0.05).
396
397 Next, we set up genetic crosses of *ABI5* overexpression line (*ABI5OE*) with *bbx30*, *bbx31*, and
398 *bbx30bbx31* to generate *bbx30ABI5OE*, *bbx31ABI5OE*, and *bbx30bbx31ABI5OE* lines,
399 respectively. Under 0µM ABA, germination and seedling establishment of all the genotypes
400 progressed at similar rates till attaining 100% (Fig. 4a, b and C, Fig. S3 a and b). In presence
401 of 1µM ABA, *ABI5OE* exhibited lower rate of seedling establishment as compared to the
402 wildtype (Fig. 4a, d and e). Interestingly, *bbx30ABI5OE*, *bbx31ABI5OE,* and
403 *bbx30bbx31ABI5OE* lines showed higher seedling establishment compared to *ABI5OE* (Fig. 4a,
404 d and e). We observed similar growth pattern among the genotypes in 0.5 and 1.5 µm ABA
405 (Fig. 4a). In 1µM ABA, *ABI5OE* showed lower % germination compared to Col-0, which was
406 partially complemented in *bbx30ABI5OE*, *bbx31ABI5OE*, and *bbx30bbx31ABI5OE* lines at 2
407 DAS (Fig. S3 c, e). All these findings together suggest that the BBX proteins BBX30 and BBX31
408 are required for ABI5-mediated seedling growth arrest. This further suggests that the
409 dependence of ABI5 on BBX30 and BBX31 is probably at the protein level, as the reduced
410 seedling establishment phenotype of seedlings constitutively expressing b *ABI5* is partially
411 rescued by the loss of *BBX30* and *BBX31.*

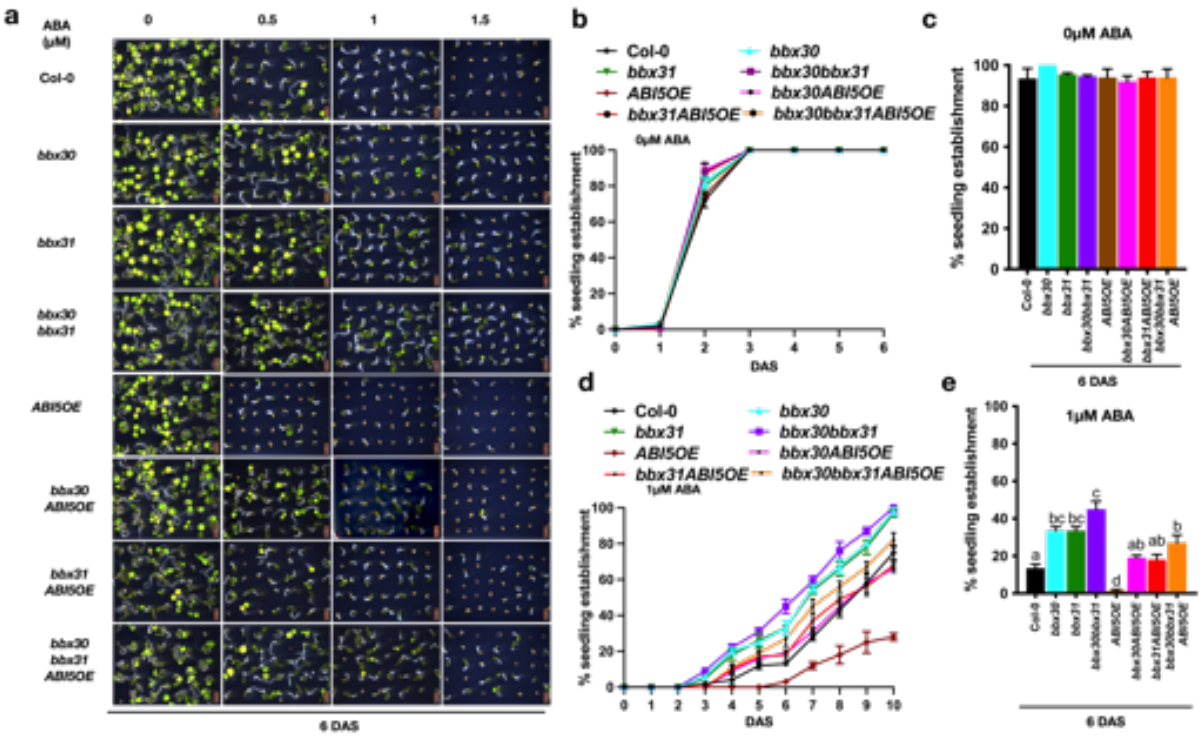


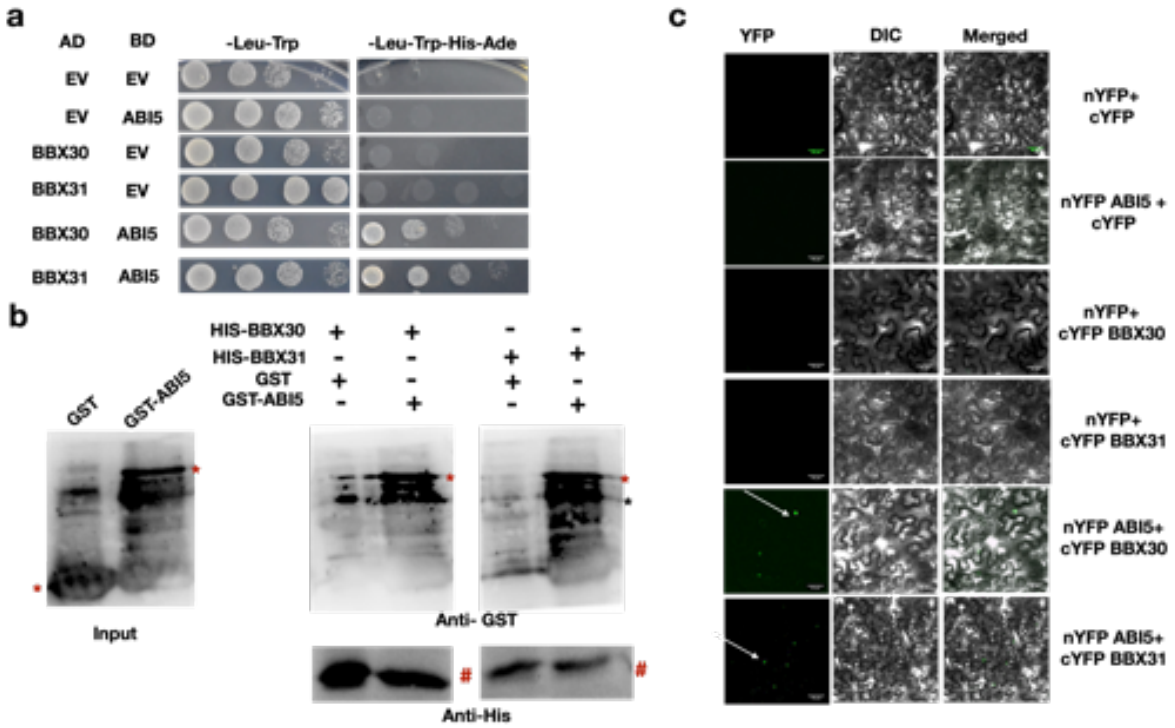
Fig. 4 BBX30 and BBX31 are required for ABI5-mediated seedling growth arrest

(a) Representative images of 6-day-old seedlings of Col-0*, bbx30, bbx31*, *bbx30bbx31, ABI5OE*, *bbx30ABI5OE, bbx31ABI5OE*, *bbx30bbx31ABI5OE* grown on a 0.5x MS plate supplemented with 0µM, 0.5µM, 1µM, 1.5µM of ABA. (b-d) % seedling establishment in (b) 0µM and (d) 1µM ABA conditions for the indicated genotypes for up to 10 days after stratification (DAS) and on day 6 (c,e). Seedlings with green, open and expanded cotyledons were counted to determine % seedling establishment. In (b-e) Error bars represent SEM of three independent experiments with 500 seeds. In (c) and (e) letters above the bar indicate the statistical groups as determined by one-way ANOVA followed by Tukey's *post hoc* test ($p < 0.05$).

ABI5 physically interacts with BBX30 and BBX31

Since constitutive overexpression of *ABI5* could not enhance ABA response in the absence of *BBX30* and *BBX31*, we hypothesized that BBX30 and BBX31 might interact with ABI5 protein and regulate its activity. In order to confirm physical interaction between ABI5 and the BBX proteins we used yeast two-hybrid, in vitro pull down and BiFC assays. Our yeast-two-hybrid assay using ABI5 as bait and either BBX30 or BBX31 as prey indicated that ABI5 can physically interact with both BBX30 and BBX31 (Fig. 5a). According to previous studies ABI5 contains conserved domains, that govern specific functions (Lopez-Molina et al., 2003; Finkelstein and

431 Lynch, 2000). We examined the interaction of BBX31 with different domains of ABI5, and
432 observed that the conserved C1 domain of ABI5 is required for the interaction with the N-
433 terminal of BBX31 (Fig. S4a and b). To further confirm the interaction between BBX30, BBX311
434 and ABI5, we performed in-vitro pull-down experiment using full length ABI5 fused to GST,
435 and BBX30 and BBX31 proteins fused to 6xHis epitope. His-tagged BBX30 and BBX31 was able
436 to pull down GST-ABI5, validating that BBX30 and BBX31 physically interact with ABI5 in vitro
437 (Fig. 5b). Furthermore, we confimed the colocalisation of the two proteins using GFP tagged
438 ABI5 and RFP tagged BBX30 and BBX31 coexpressed in *Nicotiana benthamiana* (Fig. S4c-d).
439 We also validated the interaction of these proteins in vivo using bimolecular fluorescence
440 complementation (BiFC) in *Nicotiana benthamiana* (Fig. 5c). All these data together suggest
441 that ABI5 physically interacts with BBX30 and BBX31.



442

443

444 **Fig. 5 ABI5 physically interacts with BBX30 and BBX31**

445 (a) Yeast two-hybrid assay showing the interaction between BBX30, BBX31 and ABI5. pGADT7
446 (AD) and pGBKT7 (BD) represent the *GAL4* activation domain and binding domain
447 respectively. EV indicates empty AD or BD vectors. BBX30 and BBX31 were fused to AD, while
448 ABI5 was fused with BD and tested for interaction. DDO represents the medium lacking
449 leucine and tryptophan, while QDO additionally lacks histidine and adenine. (b) In-vitro pull-

450 down assay showing the interaction between BBX30, BBX31 with ABI5. BBX30 and BBX31
451 were fused with N-terminal His tag, while ABI5 is fused with N-terminal GST tag. To check the
452 physical interaction bead-bound His-BBX30 and His-BBX31 were used to pull down GST and
453 ABI5-GST. Precipitated proteins were analysed by anti-GST and anti-His antibodies. *(red)
454 mark indicate band specific to GST-ABI5 and # indicates Anti-His corresponding band, * (black)
455 indicates nonspecific band. (c) BiFC assay showing the interaction of BBX30 and BBX31 with
456 ABI5 in planta. nYFP and cYFP represent the N-terminal and C-terminal half of yellow
457 fluorescent protein. Vectors in the indicated combinations were transformed into
458 Agrobacterium and co-infiltrated into the 3-weeks-old *N. benthamiana* leaves and imaged
459 after 36 hours. The scale bar represents 50µm.
460
461 **BBX30 and BBX31 stabilize ABI5 during post-germination development**
462 BBX30 and BBX31 belong to the group of microProteins that can sequester other proteins and
463 often regulate their stability (Wu et al., 2020; Graeff et al., 2016). Our protein-protein
464 interaction studies indicated that BBX30 and BBX31 physically interact with ABI5 (Fig. 5). To
465 understand the physiological significance of this interaction, we examined whether BBX30
466 and BBX31 can regulate the abundance of the ABI5 protein. Initially we put Col-0 and
467 *bbx30bbx31* seeds on plates containing 0µM and 1µM ABA and harvested samples every day
468 for up to 4 days after stratification and determind ABI5 accumulation by immunoblotting (Fig.
469 S5a,b). ABI5 accumulated to high levels on day 1 in both Col-0 and *bbx30bbx31* (Fig. S5a,b).
470 In -ABA conditions ABI5 accumulation in day 2, 3 and 4 samples in both the genotypes was
471 decreased (Fig. S5a,b). In presence of ABA, ABI5 accumulation was detected on day 2, 3 and
472 4 samples in Col-0 but not in *bbx30bbx31* (Fig. S5a,b). This suggests that BBX30 and BBX31
473 might regulate the stability of ABI5 especially during post-germination development. To check
474 the dynamics of ABI5 protein accumulation upon ABA treatment during post-germination
475 development, we treated germinated seeds of Col-0, *bbx30bbx31*, and *BBX31OE* with 10µM
476 ABA for 1, 6, and 12 hours and harvested samples to determine ABI5 protein levels (Fig. 6a,d).
477 ABI5 accumulation was lower in *bbx30bbx31* compared to Col-0, whereas in *BBX31OE*
478 enhanced accumulation of ABI5 was detected (Fig. 6a-d). Similar ABA treatment causes a
479 gradual increase in the ABI5 accumulation in *ABI5OE*, while this is compromised in *bbx30*
480 *bbx31 ABI5OE*, suggesting that these BBX proteins promote ABA mediated post-germination
481 ABI5 accumulation (Fig. 6e). Previous reports have shown that ABI5 protein undergoes

482 degradation via the 26S proteasomal pathway (Lee et al., 2010; Stone et al., 2006). Inhibition
483 of the 26S proteasome pathway by treating germinated seeds with MG132 for 6 hours
484 prevented the decrease in ABI5 levels in *bbx30bbx31*, suggesting that BBX30 and BBX31 could
485 promote the accumulation of ABI5 by negatively regulating its degradation (Fig. 6f). To
486 uncouple the effects of de novo translation on ABI5 levels in these genotypes, we treated the
487 germinated seeds with the translational inhibitor cycloheximide (CHX) and examined the
488 stabilization of ABI5 in Col-0*, bbx30bbx31 and BBX31OE* (Fig. 6g and h). The rate of
489 degradation of ABI5 was faster in *bbx30bbx31,* while reduced in *BBX31OE* as compared to
490 Col-0 (Fig. 6g and h). All these results together suggest that BBX30 and BBX31 promote the
491 stabilization of ABI5 during post-germination development by negatively regulating its
492 proteasome-mediated degradation.

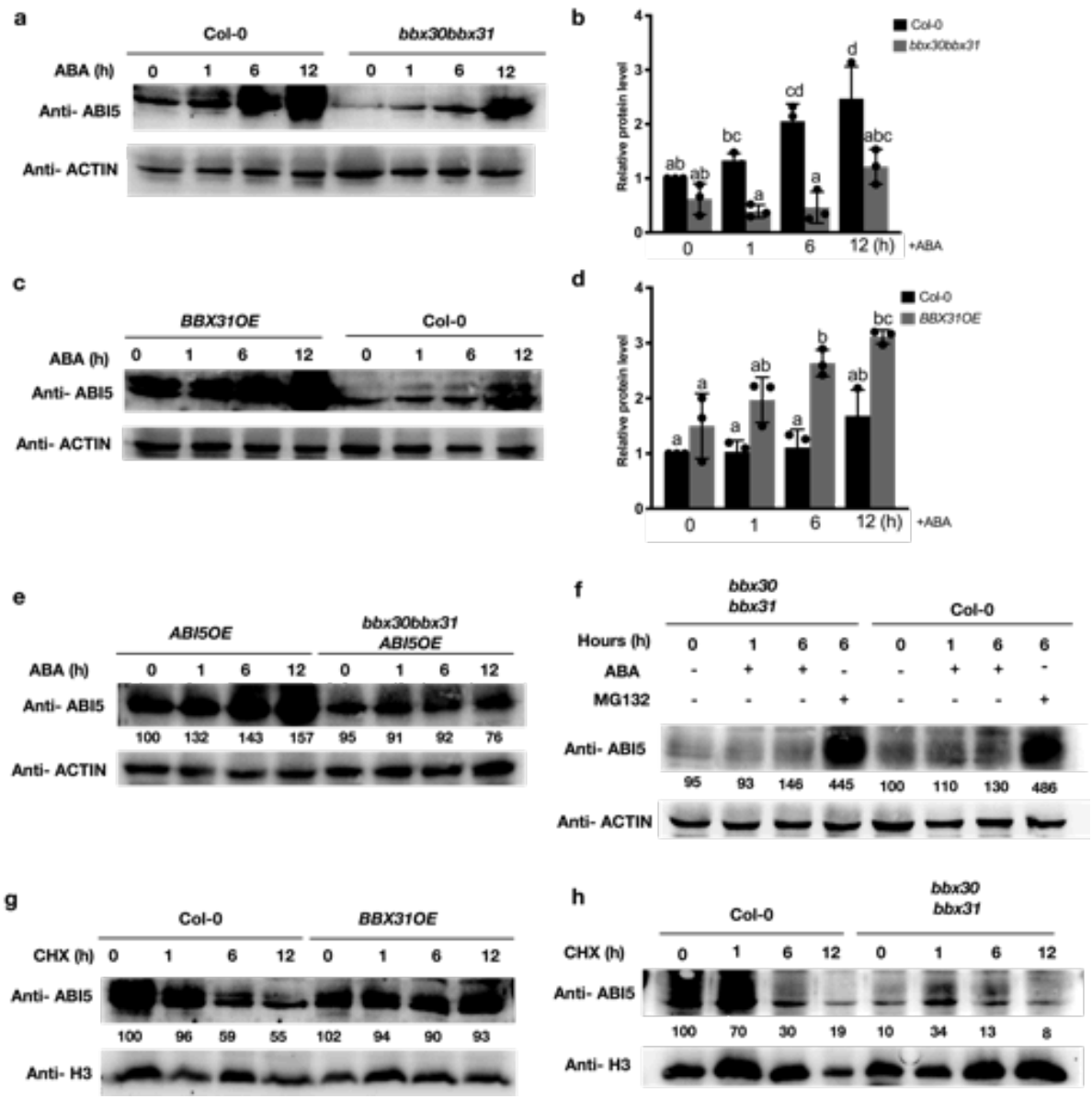


Fig. 6 BBX30 and BBX31 stabilize ABI5

(a) Immunoblot showing the abundance of ABI5 protein in Col-0 and *bbx30bbx31.* (b) Relative protein levels of ABI5 in Col-0 and *bbx30bbx31.* (c) Immunoblot showing the abundance of of ABI5 protein in *BBX31OE* and Col-0. (d) Relative protein levels of ABI5 in *BBX31OE* and Col-0. In (b) and (d) error bars represent standard deviation of two blots. Letters above the error bar indicate the statistical groups as determined by one-way ANOVA followed by Tukey's *post hoc* test ($p < 0.05$). (e) Immunoblot showing the protein level of ABI5 in *ABI5OE* and *bbx30bbx31ABI5OE* upon ABA treatment. In (a), (c) and (e) 1-day-old, germinated seeds were transferred from 0.5x MS -sucrose medium containing plates to similar plates with 10µM ABA

504 for indicated time intervals and then harvested and subjected to immunoblotting using an
505 anti-ABI5 antibody. Actin was used as an internal sample control and blotted using an anti-
506 ACTIN antibody. (f) Immunoblot showing the level of ABI5 in *bbx30bbx31* and Col-0.
507 Germinated seeds as in (a) were transferred to media containing 0.5x MS -sucrose with 10µm
508 ABA or 50µm MG132 for the indicated time. Samples were harvested and blotted using an
509 anti-ABI5 antibody. Actin was used as an internal loading sample control. (g-h) Immunoblot
510 showing the protein levels of ABI5 in Col-0, *BBX31OE*, and *bbx30bbx31* upon CHX treatment.
511 Germinated seeds were treated with 20µM ABA for 12 hours in the presence of 100µM CHX
512 and samples were harvested at different time intervals. H3 protein was used as a sample
513 loading control. Proteins were detected using Anti-ABI5 and Anti-H3 antibody respectively. In
514 (e-h) numbers below each blot indicate the relative band intensity of proteins that is
515 normalized to the loading control (ACTIN/H3). The intensity of the first band was set to 100.
516
517 **BBX30 and BBX31 enhance ABI5-mediated gene expression by promoting the binding of**
518 **ABI5 on its target promoters**
519 We now asked the question if BBX30 and BBX31 can modulate ABI5 activity. ABI5 is known to
520 directly activate its own transcription and also a range of other target genes such as *EM1* and
521 *EM6* (Chen et al., 2008c; Xu et al., 2014; Finkelstein and Lynch, 2000; Leung and Giraudat,
522 1998). First, we compared the expression level of *ABI5* and *EM6*, in Col-0, *bbx30bbx31, bbx30,*
523 *bbx31, BBX30OE, BBX31OE* under -ABA and +ABA conditions in tissues of two different
524 developmental stages - in germinated seeds that have completed the radicle emergence and
525 in developing seedlings that have started but not completed the cotyledon emergence. We
526 observed no significant difference in the expression of *ABI5* and *EM6* between the genotypes
527 in the germinated seeds (Fig. 7a and c). However, the emerging seedlings of *BBX30OE* and
528 *BBX31OE* showed higher expression of *ABI5* and *EM6* as compared to Col-0 in the presence
529 of ABA, whereas the expression was lower in the loss of function mutants (Fig. 7b and d).
530 Furthermore, we performed a transient assay in *Arabidopsis* protoplasts using luciferase gene
531 driven by *ABI5* promoter as the reporter and ABI5, BBX30, and BBX31 as effectors (Fig. 7e).
532 While the three effectors could individually induce the expression of *ABI5pro:LUC*,
533 coexpression of *ABI5, BBX30,* and *BBX31* significantly increased the luciferase activity,
534 suggesting that BBX30 and BBX31 enhance the transcriptional activation of target genes by
535 ABI5 (Fig. 7f).

536 Subsequently, we performed an electrophoretic mobility shift assay (EMSA) to test the effect
537 of BBX30 and BBX31 on the binding of ABI5 on its target promoters. BBX30 and BBX31 did not
538 show direct binding on the promoter regions of *ABI5* and *EM6* where ABI5 is known to bind
539 (Fig. 7g-i) However, the addition of BBX30 and BBX31 along with ABI5 resulted in a super shift
540 as compared to ABI5 alone, indicating that BBX30 and BBX31 might form a complex with ABI5
541 during the latter's DNA binding (Fig. 7h and i). To test whether BBX30 and BBX31 influence
542 the binding of ABI5 on its target promoters in vivo, we performed a chromatin
543 immunoprecipitation assay (ChIP) using anti-ABI5 antibody, followed by qPCR using primers
544 flanking the ABI5-binding sites of *EM1* and *EM6* promoters in 3 DAS seedlings of Col-0, *bbx30*
545 *bbx31, ABI5OE,* and *bbx30 bbx31 ABI5OE*. The enrichment of ABI5 protein on *EM1* and *EM6*
546 was decreased and increased in *bbx30bbx31* and *ABI5OE*, respectively, as compared to Col-0
547 (Fig. 7j-l). ChIP-qPCR showed the lack of ABI5 enrichment over *ACTIN* promoter used as a
548 control (Fig. S6a). Interestingly, *bbx30bbx31ABI5OE* exhibited less ABI5 protein enrichment
549 on *EM1* and *EM6* as compared to *ABI5OE*, suggesting that binding of ABI5 on the target
550 promoters is promoted by BBX30 and BBX31 (Fig. 7j-l).

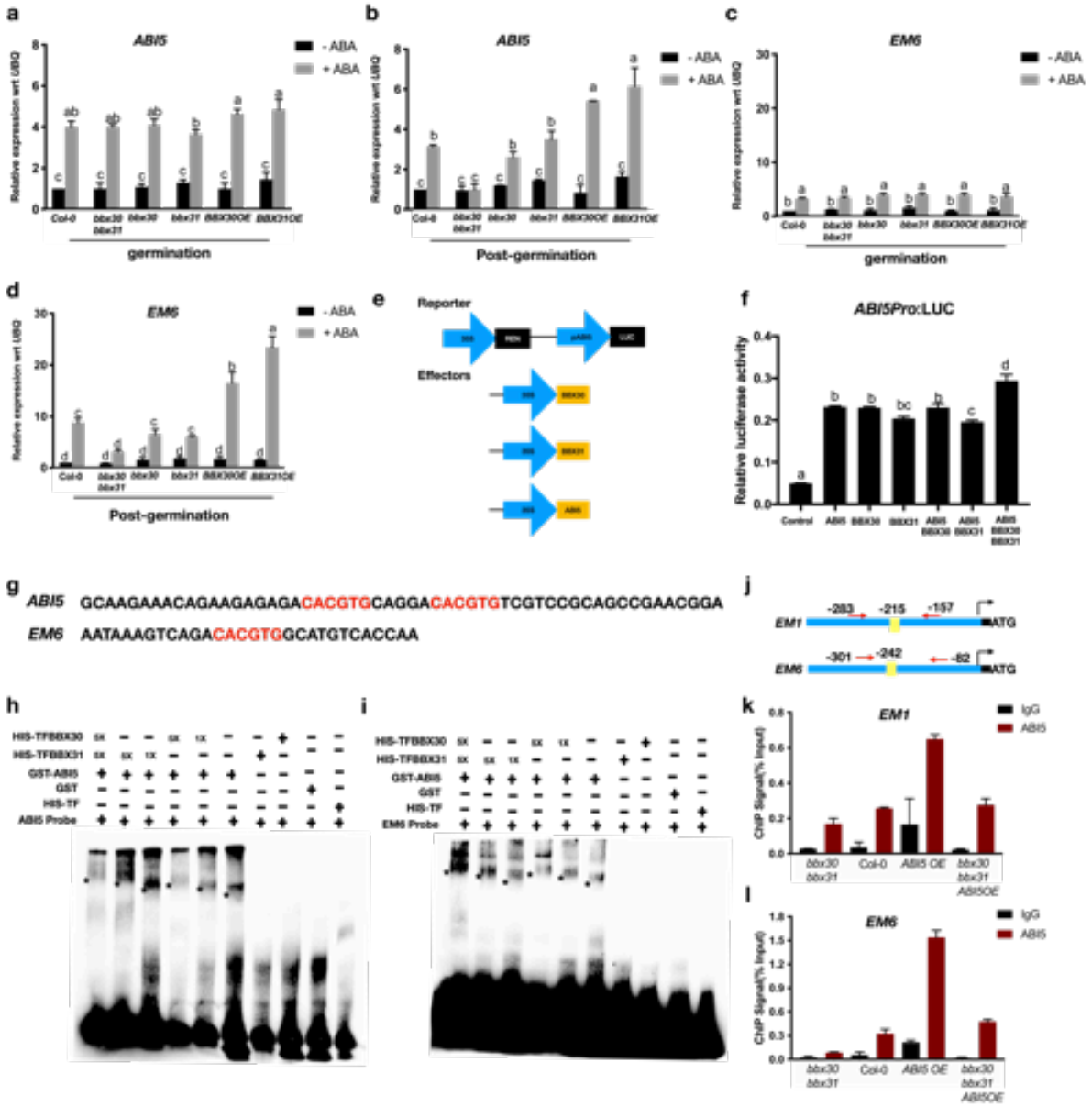


Fig. 7 BBX30 and BBX31 enhance ABI5-mediated gene expression by promoting the binding of ABI5 on its target promoters

(a-d) Relative expression of (a-b) *ABI5* and (c-d) *EM6* in Col-0, *bbx30 bbx31, bbx30, bbx31, BBX30 OE, BBX31 OE*. Seeds were inoculated on 0.5x MS -sucrose plates, with or without 1µm ABA. The seeds were harvested on the second day (germination) and the third day (post-germination) after stratification. Values are the mean of three biological replicates. (e) Schematic representation of the constructs used in the transient assay in *Arabidopsis* protoplast. The reporter construct used is based on the pGreen vector expressing *35S promoter* driven Renilla Luciferase and *ABI5*$_{pro}$ driven Luciferase. Effectors used are *ABI5, BBX30, BBX31* cloned under the constitutive *35S* promoter. (f) Relative luciferase activity,

562 showing the activation of *ABI5*$_{pro}$: LUC by the combination of proteins mentioned. Error bar
563 represents SD (n=3). (g) The sequence of promoter regions of *ABI5* and *EM6* used as probes
564 for the EMSA. The red highlighted region is the G- box type ABRE *cis*-regulatory element. (h-
565 i) EMSA showing the binding of ABI5 along with BBX30 and BBX31. BBX30 and BBX31 alone,
566 do not bind to *ABI5* and *EM6* promoters. The addition of BBX30 and BBX31 in the presence of
567 ABI5 leads to a super shift (denoted by *) of the DNA-protein complex. + and - denotes the
568 presence and absence of the probe or proteins indicated on the left. 1X and 5X represent the
569 concentration of BBX30 and BBX31. (j) Schematic representation of *EM1* and *EM6* promoter.
570 Black arrow represents the transcription start site. The yellow boxes indicates G-box type
571 ABRE *cis*-regulatory elements, and the number represents the base location of G-box type
572 elements. Red arrows denote primer binding sites for the primers used to check enrichment
573 by qPCR following ChIP. (k-l) ChIP-qPCR analysis determining the binding of ABI5 onto the
574 promoter of (k) *EM1* and (l) *EM6* in seedlings of *bbx30bbx31*, Col-0, *ABI5OE* and *bbx30*
575 *bbx31ABI5OE.* The genotypes used were grown in 1um ABA and samples were harvested on
576 the fourth day after stratification. DNA-Protein complexes were immunoprecipitated using
577 anti-ABI5 and anti-IgG antibodies (negative control). ChIP DNA was quantified using primers
578 flanking the G-box region of *EM1* and *EM6*. Letters above the error bar (a-d and f) indicated
579 the statistical groups as determined by one-way ANOVA followed by Tukey's *post hoc* test (p
580 < 0.05).
581
582 **ABI5 directly binds to the promoters of *BBX30* and *BBX31* and induces their transcription**
583 *BBX30* and *BBX31* are well characterized as light-responsive genes induced during dark to light
584 transition and are also regulated by UV-B, and the circadian clock (Yadav et al., 2019; Wu et
585 al., 2020; Graeff et al., 2016). Since our data suggest the important role of BBX30 and BBX31
586 in ABA response during post-germination development, we asked whether these genes are
587 transcriptionally regulated by ABA. We examined the expression levels of *BBX30* and *BBX31*
588 by RT-qPCR in germinated Col-0 seeds treated with 10µM ABA for 0, 1, 6 and 12 hours (Fig.
589 8a-b). ABA treatment for 1 hour results in 3-fold and 12-fold upregulation in the mRNA levels
590 of *BBX30* and *BBX31* respectively (Fig. 8a-b). Treatment with ABA for longer durations up to
591 12 hours results in the expression levels of *BBX30* and *BBX31* elevating to 4-folds and 15-folds
592 compared to no ABA treatment (0 hour) (Fig. 8a-b). The G-box type ABA response elements
593 (ABRE) present in the promoter of *BBX30* and *BBX31* are known to be the target sites of the

transcription factor ABI5 that plays crucial roles in ABA signaling. To test if *BBX30* and *BBX31* are induced by ABA in an ABI5-dependent manner, we examined their expression in *abi5-8* treated similarly as explained above for Col-0. We found that the absence of ABI5 impairs the ABA-induced upregulation of *BBX30* and *BBX31* (Fig. 8a-b). All these data suggest that after germination ABA induces the expression of *BBX30* and *BBX31* in a ABI5 dependent manner. Next, we asked the question if ABI5 can directly bind to the promoter of *BBX30* and *BBX31*. To examine this, we performed EMSA using GST-ABI5 protein and biotinylated *BBX30* and *BBX31* promoter regions as the probes. GST-ABI5 was able to bind to the G-box-containing promoter sequences of *BBX30* and *BBX31*, while the binding was abrogated in the probes containing mutated G-boxes, indicating that ABI5 directly binds on the G-box elements on the promoters of *BBX30* and *BBX31* (Fig. 8c-e). To validate the in vivo binding of ABI5 on these promoters, we also performed a ChIP-qPCR, and examined the regions of *BBX30* and *BBX31* promoters showing ABI5 enrichment. The results indicated that ABI5 indeed binds to *BBX30* and *BBX31* promoters on the regions containing the G-box type ABRE cis-regulatory elements (Fig. 8f-h). These evidences suggest that while BBX30 and BBX31 interact with ABI5 to stabilize it and promote the expression of downstream ABA responsive genes, ABI5 establishes a positive feedback loop to directly activate the transcription of *BBX30* and *BBX31* (Fig. 9).

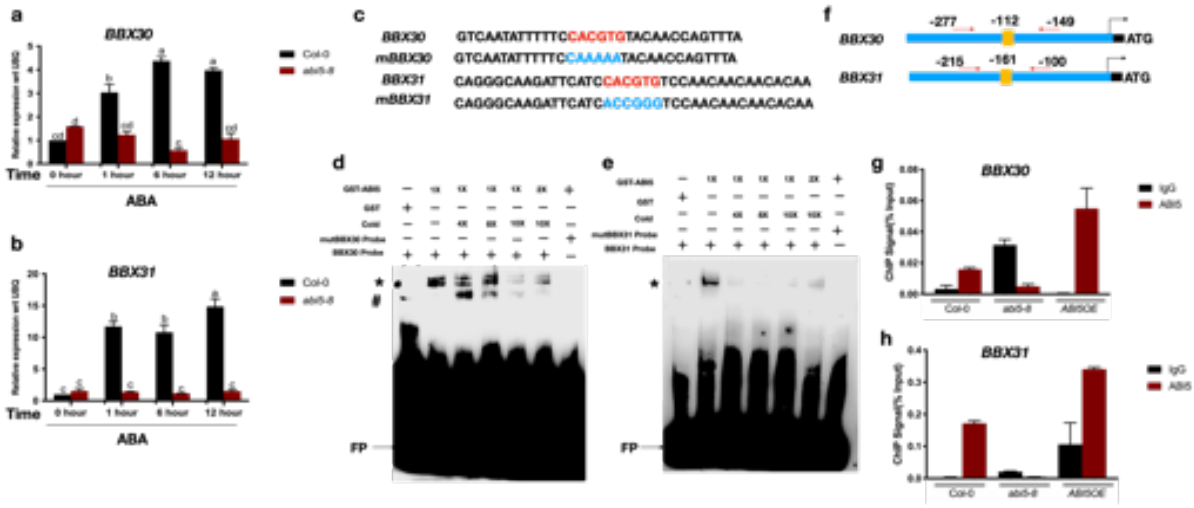


Fig. 8 ABI5 directly binds to the promoter of *BBX30* and *BBX31* and induces their transcription

(a-b) The relative expression level of (a) *BBX30* and (b) *BBX31* in the Col-0 and *abi5-8*. Seeds were germinated on 0.5xMS -sucrose conditions. 100% germinated seeds were transferred

617 to medium containing 10µM ABA. Error bars indicate SEM of two biological replicates, the
618 letters above the bars represent different statistical group. (c) Sequence of the probes used
619 for EMSA, mutated nucleotides are shown in blue colour. (d-e) EMSA showing the binding of
620 ABI5 to the promoter of *BBX30* and *BBX31*, containing the G-box domain and labelled with
621 biotin. *mutBBX30* and *mutBBX31* represent the mutated G-box, while + and - indicate
622 presence and absence; cold represents the unlabelled probes used in 4X, 8X, and 10X
623 concentrations. FP represents a free probe. # Denotes the nonspecific shift and * indicates
624 the ABI5 mediated shift. (f) Schematic representation of *BBX30* and *BBX31* promoter. Arrow
625 represents the transcription start site. The yellow boxes indicate G-box type ABRE *cis*-
626 regulatory elements, and the number represents the base location of G-box type elements.
627 (g-h) ChIP-qPCR analysis determining the binding of ABI5 onto the promoter of *BBX30* and
628 *BBX31* in seedlings of Col-0, *abi5-8,* and *ABI5OE*. The genotypes were grown in 1um ABA and
629 samples were harvested on fourth day after stratification. DNA-Protein complexes were
630 immunoprecipitated using anti-ABI5 and anti-IgG antibodies (negative control). ChIP DNA was
631 quantified using primers flanking the G-box region of *BBX30* and *BBX31*. Error bar represents
632 the the mean ± SEM of three technical replicates.
633

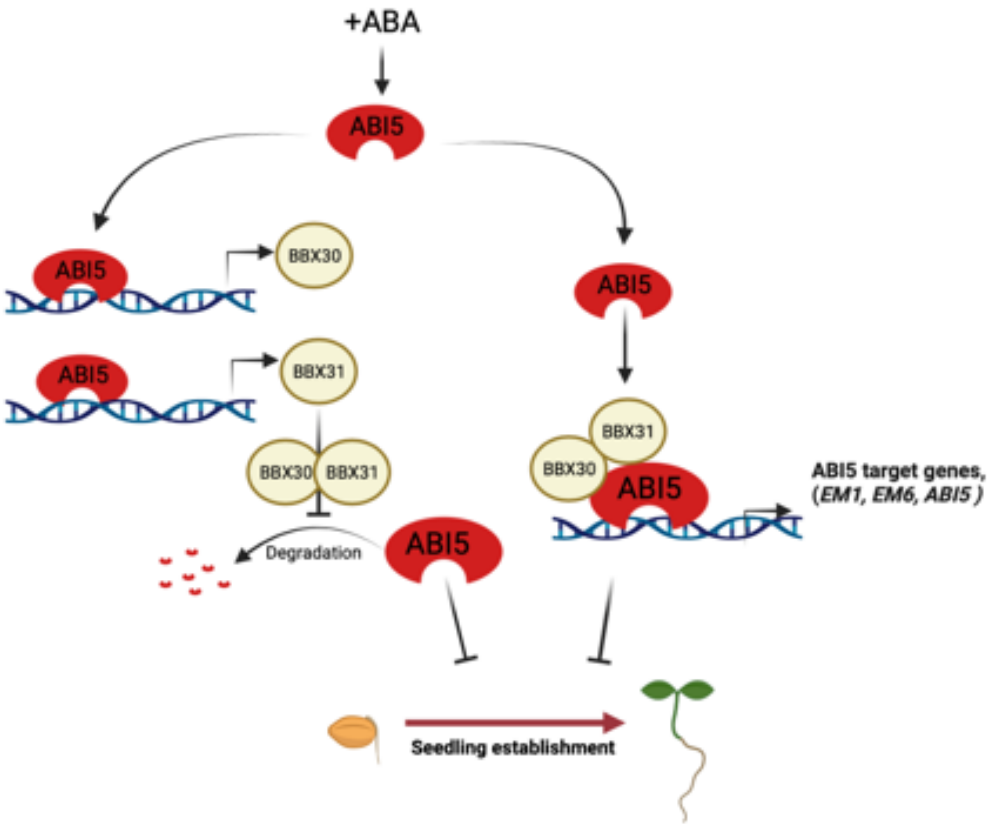


Fig. 9 Model showing BX30, BBX31 and ABI5 regulate post-germination seedling development in an interdependent manner. BBX30 and BBX31 promote the stability of ABA-induced ABI5 by inhibiting its degradation and regulating its activity to promote expression of ABI5 target genes. ABI5 on the other hand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

Discussion

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

649 and ABA signaling pathways (Xu et al., 2014; Collin et al., 2021). Several members of the light
650 signaling pathway that are modulated by the light or dark cue, feed the light information
651 either upstream or downstream of ABI5. A number of light signaling factors have been
652 identified to bind to the promoter of *ABI5* and regulate its transcription. These include
653 transcription factors like PIFs, DET1, HY5, BBX19, BBX21, FAR1 and FHY3(Sakuraba et al.,
654 2014; Penfield et al., 2010; Shi et al., 2013; Xu et al., 2014; Kang et al., 2018; Bai et al., 2019).
655 Some other light signaling regulators can modulate ABI5 activity at the protein level. This may
656 be as a result of direct physical binding with ABI5 as in the case of PIF1 or indirectly modulating
657 ABI5 stability by binding to its repressor ABD1 as mediated by COP1(Peng et al., 2022). The
658 stabilization activities of PIF1 and COP1 are limited to the dark environment. COP1 also
659 promotes ABI5 mediated downstream gene activation to suppress post-germination seedling
660 establishment (Yadukrishnan et al., 2020). PIF1 and ABI5 act in a cooperative manner to
661 activate common downstream target genes by binding to their promoters in the dark(Kim et
662 al., 2016).To prevent precocious seedling establishment ABI5 needs to be active both under
663 dark and in light. Here we identified two microProteins miP1a/ BBX31 and miP1b/BBX30 that
664 directly interact with ABI5 to stabilize it and promote its downstream activities in the light
665 (Fig. 5, 6, 7). ABI5 via a positive feedback loop regulates the transcription of miP1a/BBX31
666 and miP1b/BBX30 (Fig. 8). The microProteins and ABI5 thus mediate seedling growth arrest
667 in an interdependent manner, at least partially (Fig. 1, 2, 3, 4). This study provides a novel
668 microprotein mediated post-translational regulatory mechanism for controlling ABI5
669 accumulation and activity during post-germination seedling establishment in the absence of
670 COP1and PIFs that are deactivated in light.

671 BBX proteins are zinc-finger transcription factors that regulate numerous aspects of early
672 seedling development (Yadav et al., 2020, 2020; Song et al., 2020). In *Arabidopsis* there are
673 32 B-box proteins that are divided into five structural groups based on the domain
674 structure(Gangappa and Botto, 2014). The structural groups I-III (BBX1-BBX17) contain a CCT
675 domain at the C-terminal half which is often involved in DNA binding and transcriptional
676 regulation. BBX11 regulates the accumulation of protochlorophyllide to optimize greening
677 during de-etiolation(Job, 2020). Structural group IV contains 2 B-box domains but is devoid of
678 the CCT domain. Four members of this group (BBX20, BBX21, BBX22, BBX23) have been
679 characterized as positive regulators of photomorphogenesis while the other four (BBX18,

680 BBX19, BBX24, BBX25) repress photomorphogenic development. BBX20, BBX21, BBX22 act as
681 cofactors of HY5 to mediate light-regulated development (Bursch et al., 2020; Job et al.,
682 2018). An M6 motif identified in the C-terminus of these proteins might regulate their specific
683 developmental responses in response to light (Yadukrishnan et al., 2018). Structural group
684 five consists of 7 members (BBX26-BBX32) that contain only one B-box domain. Functional
685 characterization of these single-domain BBX proteins indicates that many of them play
686 important roles in early seedling development. BBX30 and BBX31 promote hypocotyl
687 elongation in seedlings under visible light(Yadav et al., 2019; Heng et al., 2019). HY5 inhibits
688 the transcription of *BBX30* and *BBX31* (Heng et al., 2019). Interestingly, BBX30 and BBX31
689 positively regulate *BBX28* and *BBX29* which suppresses HY5-mediated inhibition to promote
690 the transcription of *BBX30* and *BBX31* to fine-tune photomorphogenic development (Song et
691 al., 2020). BBX32 interacts with BBX21 and suppresses the promotion of light-mediated gene
692 expression by BBX21 and HY5(Holtan et al., 2011). Wu et al elegantly characterized the
693 specific role of BBX30 and BBX31 during de-etiolation (Wu et al., 2020). These two proteins
694 inhibit the dimerization of PIF3 and EIN3 to promote apical hook and cotyledon opening
695 during the dark to light transition. It is interesting to note that other group V members BBX32,
696 BBX28 and BBX29 promote BR-mediated cotyledon closure in the dark (Ravindran et al., 2021;
697 Cao et al., 2022). The functional diversity between these closely related BBX proteins needs
698 further characterization. Our study here shows that BBX30 and BBX31 inhibit post-
699 germination seedling establishment in an ABA-dependent manner. It seems that the same
700 proteins may be recruited for different functions depending on the presence or absence of
701 stress conditions. In addition to their role in modulating early seedling development, BBX30
702 and BBX31 also regulate flowering and BBX31 promotes UV-B stress tolerance(Yadav et al.,
703 2019; Graeff et al., 2016).

704 MicroProteins miP1a/BBX31 and miP1b/BBX30 are 121 and 117 amino acids long respectively
705 and show 65.5% sequence identity(Graeff et al., 2016). microProteins form homodimers,
706 heterodimers, or multimeric complexes by interacting with the protein-protein interaction
707 domains of their targets. As of now, not more than 50 miPs have been functionally
708 characterized in plants. In most of these cases, the microProteins repress the activity of their
709 targets(Staudt and Wenkel, 2011). In fact, miP1a and miP1b have been previously shown to
710 form a repressor complex together with CO and TOPLESS to inhibit flowering(Graeff et al.,

711 2016). This microProtein duo also inhibits the oligomerization of PIF3 and EIN3 to repress
712 their transcriptional activation potential (Wu et al., 2020). Here we report that miP1a/BBX31
713 and miP1b/BBX30 physically interact with ABI5, stabilize it and promote its binding to the
714 downstream targets to activate their expression (Fig. 5,6,7). The versatility in the mode of
715 action of the same miPs probably depending on the developmental stage of the plant and the
716 environmental conditions is intriguing. During dark to light transition, miP1a/BBX31 and
717 miP1b/BBX30 promote seedling de-etiolation, however, in the presence of ABA, these miPs
718 arrest seedling development. What is the basis of this differential regulation in the presence
719 or absence of stress is yet to be discovered. Our data shows that ABA induces the expression
720 of *miP1a/BBX31* and *miP1b/BBX30* in a ABI5 dependent manner (Fig. 8). ABI5 binds to the
721 G-box motif present in the promoters of *miP1a/BBX31* and *miP1b/BBX30* to activate their
722 expression (Fig. 8). Previously HY5 has been shown to bind to the G-box of *BBX30* and *BBX31*
723 to repress their transcription in light(Heng et al., 2019; Yadav et al., 2019). However this
724 differential transcriptional regulation of the miPs is unlikely to account for their varied post-
725 translational activity in presence or absence of ABA. The upregulation of *miP1a/BBX31* and
726 *miP1b/BBX30* expression is extremely rapid and transient happening within the 1 hour of the
727 transfer from dark to light (Wu et al., 2020). The inhibitory activity of miP1a/BBX31 and
728 miP1b/BBX30 in preventing PIF3 and EIN3 functional oligomer formation also seems to be
729 transient as PIF3 and EIN3 are eventually degraded by light. It is possible that the miPs might
730 disengage from this transient activity in the presence of stress. It would be interesting to see
731 what happens to the allosteric deactivation of PIF3 and EIN3 by miP1a and miP1b in presence
732 of ABA. The cooperative action of miP1a and miP1b in interacting with its targets is also a
733 unique feature of this regulatory module that needs further characterization. In several
734 eukaryotes, miPs regulate transitions in the cell cycle and circadian clock. In yeast, the
735 microProtein Nrs1 rewires the transcriptional machinery to regulate G1/S transition under
736 nutrient stress conditions. This study and some previous reports indicate the role of
737 miP1a/BBX31 and miP1b/BBX30 in modulating the transition from seed to seedling and
738 vegetative to flowering state (Wu et al., 2020; Graeff et al., 2016). In the plant kingdom, miPs
739 are generally present in dicotyledonous flowering plants and might have evolved to modulate
740 these crucial transitions that regulate plant life and productivity (Graeff et al., 2016). The
741 identification of light regulated microProteins in modulating ABA sensitivity during the early
742 establishment phase, might open the doors for optogenetic manipulation of these versatile

743 regulators. In the future, synthetic miPs may be used as molecular brakes to postpone
744 development during unfavourable conditions to ensure plant survival.

745

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757 **Author contributaion:**

758 SD and DS conceived the project. DS planned and performed all the experiments. SD wrote
759 the manuscript with help from DS. DS and SD analysed the data and helped preparing the
760 final manuscript.

761 **Data Availability Statement**
762 The data that supports the findings of this study are available in the supplementary material
763 of this article.
764

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