bHLH11 negatively regulates Fe homeostasis by its EAR motifs recruiting corepressors in Arabidopsis

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Running title: bHLH11 acts an active repressor

One-sentence summary: bHLH11 interacts with and inhibits transcriptional activation ability of bHLH IVc TFs via its EAR motifs recruiting the TOPLESS/TOPLESS-RELATED corepressors to finetune Fe homeostasis in Arabidopsis.

31

32 **ABSTRACT**

33

34 Iron (Fe) homeostasis is essential for plant growth and development. Although
 35 tremendous progress has been made in understanding the maintenance of Fe
 36 homeostasis in plants, the underlying molecular mechanisms remain elusive.
 37 Recently, bHLH11 was reported to function as a negative regulator. However,
 38 the molecular mechanism by which bHLH11 regulates Fe homeostasis is
 39 unclear. Here, we generated two *bhlh11* loss-of-function mutants which
 40 displayed the enhanced sensitivity to excessive Fe. bHLH11 is located in the
 41 cytoplasm and nucleus due to lack of a nuclear location signal sequence, and
 42 its interaction partners, bHLH IVc transcription factors (TFs) (bHLH34,
 43 bHLH104, bHLH105 and bHLH115) facilitate its nuclear accumulation. bHLH11
 44 exerts its negative regulation function by recruiting the corepressors
 45 TOPLESS/TOPLESS-RELATED. Moreover, bHLH11 antagonizes the
 46 transactivity of bHLH IVc TFs towards bHLH Ib genes (*bHLH38*, *bHLH39*,
 47 *bHLH100* and *bHLH101*). This work indicates that bHLH11 is a crucial
 48 component of Fe homeostasis signaling network, playing a pivotal role in the
 49 fine-tuning of Fe homeostasis.

50

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INTRODUCTION

Iron (Fe) is an indispensable microelement for plant growth and development. Plants acquire Fe from the soil, which has low concentrations of Fe available, especially in alkaline environments (Jeong and Guerinot, 2009). As about one-third of the world's cultivated land is calcareous (alkaline), iron deficiency is common for plants. Fe functions in many physiological processes, such as photosynthesis, respiration, hormone biosynthesis, and nitrogen fixation. Fe deficiency causes symptoms including delayed growth and leaf chlorosis and can affect the yield and nutritional quality of crops. Although Fe is required for plant growth and development, excess Fe can be toxic to plants because Fe can cause the production of reactive oxygen radicals that are harmful to plant cells (Quinet et al. 2012). Therefore, maintaining Fe homeostasis in plant cells is crucial for their normal growth and development.

Plants have evolved a set of molecular mechanisms for iron absorption, transport, distribution, and storage that ensure appropriate Fe concentrations in cells under low Fe availability. Dicotyledonous and non-gramineous monocotyledonous plants take up Fe using a reduction strategy (strategy I). In *Arabidopsis thaliana*, this strategy involves rhizosphere acidification, ferric iron reduction, and ferrous iron transport. H⁺-ATPases such as the P-type ATPase AHA2/AHA7 release protons into the soil, which improves the solubility of Fe in the soil (Santi and Schmidt, 2009; Kobayashi and Nishizawa, 2012). Then, the root surface Fe chelate reductase FERRIC REDUCTION OXIDASE2 (FRO2) catalyzes the reduction of Fe³⁺ to Fe²⁺ (Robinson et al. 1999). Transporters such as IRON-REGULATED TRANSPORTER1 (IRT1) transport Fe²⁺ into roots (Henriques et al. 2002; Varotto et al. 2002; Vert et al. 2002). By contrast, gramineous plants employ a chelation strategy (strategy II) in which high-affinity Fe chelators of the mugineic acid family, also known as phytosiderophores, are secreted into the rhizosphere and facilitate the uptake of the Fe³⁺-phytosiderophore complex. Recent studies suggest that secretion

82 of Fe-chelating compounds is also important for the survival of
83 non-gramineous plants such as Arabidopsis in alkaline soil (Rodríguez-Celm
84 et al. 2013; Schmid et al. 2014; Fourcroy et al. 2014; Fourcroy et al. 2016;
85 Siwinska et al. 2018; Tsai et al. 2018).

86 To maintain Fe homeostasis, plants must sense the environmental Fe
87 concentration and fine-tune the expression of Fe uptake-associated genes
88 accordingly. BRUTUS (BTS) interacts with the basic helix-loop-helix
89 transcription factors bHLH105 and bHLH115 and promotes their degradation
90 (Selote et al. 2015). bHLH105 and bHLH115 belong to the bHLH IVc group,
91 which contains four members. The other two members are bHLH34 and
92 bHLH104. These four members regulate the expression of *FER-LIKE*
93 *IRON-DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT)*, bHLH Ib
94 genes (*bHLH38*, *bHLH39*, *bHLH100*, and *bHLH101*) and *POPEYE (PYE)*
95 (Zhang et al. 2015; Li et al. 2016; Liang et al. 2017). Recently, three studies
96 characterized the functions of bHLH121 (Kim et al. 2019; Gao et al. 2020; Lei
97 et al. 2020), and Lei et al. (2020) found that *bHLH121* is also directly regulated
98 by bHLH IVc. FIT interacts with bHLH Ib TFs and they synergistically promote
99 the expression of Fe-uptake associated genes *IRT1* and *FRO2* (Yuan et al.
100 2008; Wang et al. 2013). PYE and bHLH11 are negative regulators of Fe
101 homeostasis (Long et al. 2010; Tanabe et al. 2019). bHLH105/ILR3 also
102 functions as a negative regulator when it interacts with PYE (Tissot et al. 2019).
103 In contrast, bHLH121 is required for activation of numerous Fe deficiency
104 responsive genes (Kim et al. 2019; Gao et al. 2020; Lei et al. 2020). Moreover,
105 both bHLH 121 and PYE interact with bHLH IVc to regulate Fe homeostasis in
106 Arabidopsis (Long et al. 2010; Selote et al. 2015; Kim et al. 2019; Tissot et al.
107 2019; Gao et al. 2020; Lei et al. 2020). There is also a similar Fe deficiency
108 response signaling network in rice (Ogo et al. 2007; Kobayashi 2013, 2019;
109 Zhang et al. 2017, 2020; Wang et al. 2020; Li et al. 2020).

110 In the present study, we characterized the roles of bHLH11 in the
111 maintenance of Fe homeostasis in Arabidopsis. bHLH11 recruits the

transcriptional corepressors TOPLESS/TOPLESS-RELATED (TPL/TPR) to exert its transcriptional repression function. bHLH11 is localized in both cytoplasm and nucleus and is exclusively in the nucleus when bHLH IVc TFs are abundant. bHLH11 also interacts with and inhibits bHLH IVc TFs.

RESULTS

Loss-of-function of *bHLH11* impairs Fe homeostasis

Recently, it was reported that the overexpression of *bHLH11* leads to the enhanced sensitivity to Fe deficiency (Tanabe et al. 2019). Here, we also confirmed this result (Figure S1). To further explore the functions of bHLH11, we attempted to isolate two T-DNA insertion mutants from the stocks (WiscDsLoxHs168_11D and SAIL_196_A11). However, no T-DNA was identified within the *bHLH11* gene in the WiscDsLoxHs168_11D; and *bHLH11* mRNA was slightly increased in the homozygous line of SAIL_196_A11 with a T-DNA in the promoter region (data not shown). Although the homozygous line of SAIL_196_A11 was lethal according to the recent study (Tanabe et al. 2019), we observed that they developed as well as the wild type. Thus, we employed the CRISPR-Cas9 system to edit *bHLH11*. Two single guide RNAs were designed to specifically target exons 4 and 3 of *bHLH11* and respectively integrated into the binary vector with a Cas9 (Liang et al. 2016) which were then used for transformation of the wild-type plants. We identified two homozygous mutants (*bhlh11-1* and *bhlh11-2*), the former containing a 1-bp insertion in exon 4 and the latter containing a 2-bp deletion in exon 3 (Figure S2), both of which caused a frameshift mutation in the bHLH domain. When grown on Fe0 or Fe100 media, no visible difference was observed between the *bhlh11* mutants and WT (Figure 1A). By contrast, when grown on Fe300 media, the *bhlh11* mutants produced small shoots (Figure 1A, B). Fe content analysis suggested that the Fe concentration of *bhlh11* mutants was higher

142 than that of the WT (Figure 1C). These data suggest that the loss-of-function of
143 *bHLH11* leads to the enhanced sensitivity to Fe excess.

144 To further investigate the effect of *bHLH11* on the Fe signaling network, we
145 examined the expression of several Fe homeostasis associated genes (Table
146 1). *IRT1* and *FRO2* were markedly lower in the *bHLH11-OX* plants than in the
147 WT, whereas *BTS*, *PYE*, (*IRON MAN 1*) *IMA1*, *bHLH 1b*, were higher. By
148 contrast, *BTS*, *PYE*, *IMA1*, *bHLH 1b*, and *FIT*, were expressed at higher levels
149 in the *bhlh11* mutants than in the WT. These data suggest that loss-of-function
150 of *bHLH11* impairs Fe homeostasis.

151

152 ***bHLH11* expression and subcellular localization**

153 To investigate the response of *bHLH11* to Fe status, RT-qPCR was used to
154 determine the expression of *bHLH11* in response to Fe status, showing that
155 *bHLH11* mRNA increased in the roots with an increase of Fe concentration in
156 the growth media (Figure 2A), which is in consistence with the recent study
157 (Tanabe et al. 2019). We next examined the response of *bHLH11* proteins to
158 Fe status. One-week-old *bHLH11* overexpression plants grown on Fe100
159 media were transferred to Fe0 or Fe300 media, and root samples were
160 harvested after 1, 2, and 3 days. Immunoblot analysis showed that *bHLH11*
161 increased with an increase in Fe concentration and decreased with a decrease
162 in Fe concentration (Figure 2B).

163 Several Fe-homeostasis associated *bHLH* TFs were found outside the
164 nucleus (Gratz et al. 2019; Trofimov et al. 2019; Lei et al. 2020; Wang et al.
165 2020; Liang et al. 2020). To determine the subcellular localization of *bHLH11*,
166 we generated the 35S:*bHLH11*-mCherry construct, in which the mCherry tag
167 was fused in frame with the C terminus of *bHLH11*. When this construct was
168 transiently expressed in tobacco leaves, mCherry was mainly observed in the
169 cytoplasm and nucleus, which is very similar to that of free mCherry (Figure
170 2C). The cytoplasmic localization of *bHLH11* was unexpected because
171 transcription factors are known to function in the nucleus. Thus, we examined

whether bHLH11 can be retained in the cytoplasm due to a lack of a nuclear localization signal (NLS). NLS prediction was conducted by cNLS Mapper with a cutoff score = 4 (Kosugi et al. 2009; http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_y.cgi). No NLS was found in bHLH11 (Figure S3). When bHLH11 was fused with NLS-mCherry, which contains an NLS from the SV40 virus, bHLH11-NLS-mCherry was exclusively localized in the nucleus (Figure 2C). These data suggest that lack of an NLS causes the cytoplasmic localization of bHLH11.

180

181 **bHLH11 interacts with bHLH IVc TFs in the nucleus**

182 Considering that TFs usually functions in the nucleus and an NLS allowed
183 bHLH11 to remain in the nucleus, we hypothesized that bHLH11 might be
184 recruited to the nucleus by its nuclear-localized interaction partners. Recent
185 studies revealed that bHLH121, the closest homolog of bHLH11, interacts with
186 bHLH IVc TFs (Kim et al. 2019; Gao et al. 2020; Lei et al. 2020). Therefore, we
187 employed the yeast two-hybrid system to test whether bHLH11 interacts with
188 bHLH IVc TFs. The bHLH11 protein was fused with the GAL4 DNA binding
189 domain in the pGBKT7 vector as the bait (BD-bHLH11). bHLH IVc TFs were
190 cloned to the pGADT7 vector as the preys. As expected, bHLH11 interacts with
191 all four bHLH IVc TFs in yeast (Figure 3A).

192 To confirm that bHLH IVc TFs interact with bHLH11 in plant cells, we
193 employed the tripartite split-GFP system (Liu et al. 2018). The GFP10
194 fragment was fused with bHLH IVc proteins in their N-terminus (GFP10-bHLH
195 IVc) and the GFP11 was fused with bHLH11 in the C-terminus
196 (bHLH11-GFP11). When GFP10-bHLH IVc and bHLH11-GFP11 were
197 transiently co-expressed with GFP1-9 in tobacco leaves, the GFP signal was
198 visible in the nucleus of transformed cells (Figure 3B). By contrast, the other
199 combinations did not result in a GFP signal in the cells (Figure 3B).

200 We next performed a coimmunoprecipitation (Co-IP) assay to confirm the
201 interactions between bHLH IVc TFs and bHLH11 (Figure 3C). MYC tag-fused

bHLH IVc TFs and HA tag-fused bHLH11 were transiently co-expressed in tobacco leaves. The total proteins were incubated with MYC antibody and A/G-agarose beads and then separated on SDS-PAGE for immunoblotting with HA antibody. Consistent with the results from the yeast two-hybrid and tripartite split-GFP assays, bHLH IVc and bHLH11 were present in the same protein complex. These data suggest that bHLH IVc TFs physically interact with bHLH11.

209

bHLH IVc TFs affect the subcellular localization of bHLH11

Having confirmed that bHLH11 interacts with bHLH IVc TFs in the nucleus, we wondered whether the bHLH IVc TFs have an impact on the subcellular localization of bHLH11. When any one of these four GFP tagged proteins was respectively co-expressed with bHLH11-mCherry, bHLH11-mCherry accumulated exclusively in the nucleus (Figure 4A). By contrast, co-expression of the free GFP did not affect the subcellular localization of bHLH11-mCherry (Figure 4A).

To further confirm the distribution of bHLH11 in the cytoplasm and nucleus, we used immunoblot to measure the expression of the bHLH11 protein. As shown in Figure 4B, bHLH11 protein was detected both in the nucleus and cytoplasm, and both its nuclear and cytoplasmic counterparts were responsive to Fe status.

223

bHLH11 antagonizes the transactivity of bHLH IVc TFs

The bHLH Ib genes are activated directly by the bHLH IVc TFs (Zhang et al. 2015; Li et al. 2016; Liang et al. 2017). Our expression analyses also suggested that the bHLH Ib genes were upregulated in the *bhlh11* mutants, implying that bHLH11 is a negative regulator of bHLH Ib genes. Because bHLH11 interacts with the bHLH IVc TFs, we proposed that bHLH11 could antagonize the functions of the bHLH IVc TFs. To confirm this hypothesis, transient expression assays were conducted in Arabidopsis protoplasts (Figure

232 5A). The reporter construct *ProbHLH38:LUC*, in which the LUC reporter was
233 fused with the promoter of *bHLH38*, and different effectors in which the 35S
234 promoter was used to drive GFP, bHLH11 or bHLH IVc, were used in the
235 transient assays. Compared to GFP, bHLH IVc TFs activated the expression of
236 *ProbHLH38:LUC*, whereas bHLH11 had no significant effect. When bHLH11
237 and bHLH IVc were co-expressed, the the LUC/REN ratio declined significantly.
238 These data suggest that bHLH11 inhibits the transactivity of bHLH IVc TFs
239 towards *bHLH38*.

240 To further investigate whether bHLH11 inhibits the functions of bHLH IVc
241 TFs by direct protein–protein interaction, we employed the *pGAL4* promoter. In
242 the reporter construct, GFP fused with an NLS sequence was driven by *pGAL4*
243 containing the minimal CaMV 35S promoter with five repeats of the GAL4
244 binding motif (Figure 5B). In the effectors, the DNA binding domain (BD) of
245 GAL4 was fused in frame with either bHLH104 or bHLH105 and driven by the
246 35S promoter. Consistent with the fact that bHLH IVc TFs are transcriptional
247 activators, the chimeric BD-bHLH104 or bHLH105 activated the expression of
248 *GFP*. When bHLH11 was co-expressed with BD-bHLH104 or BD-bHLH105,
249 the expression of *GFP* was significantly repressed. These data suggest that
250 bHLH11 antagonizes the transcriptional activation ability of bHLH IVc TFs
251 through direct protein interaction.

252 Although bHLH11 antagonizes the functions of bHLH IVc TFs, its
253 overexpression caused the increase expression of bHLH Ib genes. We
254 reasoned that the disrupted Fe homeostasis and severe growth inhibition may
255 account for the upregulation expression of bHLH Ib genes in *bHLH11-OX*
256 plants. To confirm our hypothesis, we generated transgenic plants containing a
257 *pER8-bHLH11* construct, in which the *HA-bHLH11* fusion gene was under the
258 control of an inducible promoter, activated by estradiol. Under control
259 conditions, the *bHLH11* transcript level was similar between the WT and
260 *pER8-bHLH11*. After treatment with estradiol, the *bHLH11* gene was
261 overexpressed in the *pER8-bHLH11* plants with no obvious change in the WT

(Figure S4A). As expected, the *pER8-bHLH11* plants displayed enhanced sensitivity to Fe deficiency when grown on Fe0 + estradiol media (Figure S4B). To examine the expression of Fe deficiency-responsive genes, plants grown on Fe100 media were shifted to Fe0 media and Fe0 + estradiol media for 3 days. We found that the expression of bHLH Ib genes was downregulated in the *pER8-bHLH11* plants after treatment with estradiol (Figure 5C).

Taken together, our data suggest that bHLH11 antagonizes the transcriptional activation ability of bHLH IVc TFs.

270

bHLH11 functions as a transcriptional repressor by recruiting TOPLESS/TOPLESS-RELATED corepressors

Considering that bHLH11 negatively regulated the expression of Fe deficiency-responsive genes, we proposed that bHLH11 is a transcriptional repressor. To investigate how bHLH11 represses transcription, we analyzed its protein sequence and found two typical ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motifs (LxLxL) in the C-terminal region of bHLH11 (Figure 6A;). The EAR motif is a characteristic of proteins interacting with the TPL/TPRs which function as transcription corepressors (Szemenyei et al. 2008; Pauwels et al. 2010; Causier et al. 2012). Thus, we determined whether bHLH11 interacts with TPL/TPRs. Yeast two-hybrid assays indicated that bHLH11 interacts with TPL/TPRs (Figure 6B). To further investigate whether the EAR motifs are required for the interaction, the various EAR-mutated versions, bHLH11m1, bHLH11m2, and bHLH11dm, were tested for the interaction with TPL/TPRs. As shown in Figure 6B, the interaction between bHLH11 and TPL/TPRs was dependent on the EAR motifs, as mutation of both EAR motifs abolished the interaction between bHLH11 and TPL/TPRs.

To investigate whether the EAR motifs are required for the repressor function of bHLH11, we conducted reporter-effector transient expression assays in which bHLH105 was used as an effector to activate

292 *ProbHLH38-LUC*. We compared the effects of GFP, bHLH11, bHLH11dm, and
 293 bHLH11dm-VP16 (VP16, an established activation domain) on bHLH105
 294 (Figure 6C). Compared to the significant repression effect of bHLH11 on
 295 bHLH105, bHLH11dm had no significant effect, whereas bHLH11dm-VP16
 296 enhanced the transactivation function of bHLH105 (Figure 6D). These data
 297 suggest that bHLH11 functions as a transcriptional repressor and that this
 298 function is dependent on its EAR motifs.

299 To assess the consequences of disrupting the repressor functions of
 300 bHLH11 *in vivo*, we generated *bHLH11dm-VP16* transgenic plants. We
 301 hypothesized that *bHLH11dm-VP16* overexpression would not only be able to
 302 inhibit the functions of bHLH11 by competing with the endogenous bHLH11,
 303 but might also activate the target genes of bHLH11. Indeed, *bHLH11dm-VP16*
 304 plants showed enhanced tolerance to Fe deficiency compared to *bHLH11-OX*
 305 plants (Figure 6E). Expression analysis suggested that overexpression of
 306 *bHLH11dm-VP16* promotes the expression of *IRT1* and *FRO2* (Figure S5).
 307 Therefore, the EAR motifs are needed for the repression function of bHLH11.

308

309 DISCUSSION

310

311 Plants sense Fe-deficient environments and activate a signal transduction
 312 cascade that ultimately results in the transcriptional regulation of downstream
 313 effector genes of the Fe uptake system. The expression of Fe
 314 homeostasis-associated genes is tightly regulated by Fe availability, including
 315 environmental Fe availability and local Fe availability in developing tissues and
 316 organs. However, this mechanism is not an on-off process but rather a
 317 fine-tuned one, with multiple layers of transcription regulations. Considerable
 318 progress has been made in deciphering the signal transduction pathways that
 319 maintain Fe homeostasis, leading to the identification of many signaling
 320 components. Here, we show that bHLH11 acts as an active repressor by
 321 recruiting TPL/TPRs. bHLH11 contributes to Fe homeostasis through a

sophisticated mechanism that involves physical interaction with bHLH IVc TFs, which down-regulates their transcription activation capacity.

We demonstrated that bHLH11 negatively regulates Fe uptake at the molecular and physiological level. The *bHLH11-OX* plants had reduced Fe concentration whereas its mutants had elevated Fe concentration (Figure 1C; Figure S1D), which is positively correlated with the expression *IRT1* and *FRO2*. It is well known that FIT interacts with bHLH Ib TFs to control the expression of *IRT1* and *FRO2*. Although bHLH Ib genes were upregulated and *FIT* was not affected in the *bHLH11-OX* plants, the expression of *IRT1* and *FRO2* was extremely inhibited (Table 1). Recently, Tanabe et al. (2019) reported that *bHLH11* overexpression inhibits the expression of both *FIT* and *IRT1/FRO2*, and concluded that bHLH11 negatively regulates *IRT1* and *FRO2* in a FIT-dependent manner (Tanabe et al. 2019). However, we did not find the significant response of *FIT* to *bHLH11* overexpression. Therefore, it is plausible that bHLH11 directly represses the expression of *IRT1* and *FRO2*. Recently, Gao et al. (2020) found that bHLH11 interacts with itself in yeast, implying that bHLH11 may function as a homodimer to regulate its target genes.

Two types of transcriptional repressors exist: active and passive (Krogan and Long, 2009). Generally, active repressors repress transcription by recruiting transcriptional repression components, whereas passive repressors indirectly influence transcription by competitively interfering with activators. The TPL/TPRs are a class of corepressors (Causier et al. 2012). Our work confirmed that bHLH11 interacts with TPL/TPRs and negatively regulates gene expression, suggesting that bHLH11 functions as an active repressor. The observation that the EAR motif (LxLxL) is conserved in bHLH11 homologs across different plant species, such as maize (*Zea mays*), rice, and *Brassica rapa* (Figure S6), implies that different plant species employ a conserved repression strategy to fine-tune Fe homeostasis.

The antagonistic regulation between positive and negative transcription

352 factors is prevalent in plants. For example, the transcription factors MYC2,
353 MYC3, and MYC4 as well as bHLH3, bHLH13, bHLH14, and bHLH17
354 antagonistically regulate jasmonic acid (JA) signaling (Fernandez-Calvo et al.
355 2011; Song et al. 2013). In Fe homeostasis signaling, the bHLH IVa TFs
356 (bHLH18, bHLH19, bHLH20, and bHLH25) antagonize the activity of the bHLH
357 Ib TFs in regulating FIT protein stability under Fe deficiency (Cui et al. 2018).
358 Here, we show the antagonistic regulation between bHLH11 and bHLH IVc
359 TFs. In addition to transcriptional regulation, the protein degradation is another
360 type of regulation in Fe homeostasis signaling. As reported previously,
361 bHLH105 and bHLH115 are degraded by BTS via the 26S proteasome
362 pathway (Selote et al. 2015). We found that bHLH11 protein was degraded
363 under Fe deficient conditions (Figure 2B), which may benefit plants by
364 alleviating the repression of bHLH11 to Fe uptake associated genes. These
365 coordinated regulations of transcription and post-transcription may help plants
366 adapt to their various Fe-nutrition habitats.

367 bHLH11 has no canonical NLS sequence (Figure S3). We showed that
368 bHLH11 exists in the nucleus and cytoplasm and it accumulates in the nucleus
369 with the assistance of its nuclear partners bHLH IVc TFs (Figure 4A). This
370 transcription factor-dependent nuclear localization of bHLH11 might contribute
371 to the maintenance of Fe homeostasis. bHLH11 inhibits the activation ability of
372 bHLH IVc TFs and restricts the expression of Fe uptake-associated genes.
373 The repressor function of bHLH11 may help plants avoid Fe toxicity and adapt
374 to environments with an Fe excess by reducing the rate of Fe uptake. This
375 putative protein shuttling strategy enables plants to respond quickly to Fe
376 fluctuation.

377 Transcriptional activation of Fe deficiency-responsive genes occurs
378 downstream of the bHLH IVc TFs. However, this mechanism may not be an
379 on-off process. We show that bHLH11 contributes to Fe homeostasis via a
380 sophisticated mechanism. As a negative transcription factor, bHLH11 exerts its
381 repressor function by its EAR motifs recruiting the TPL/TPRs (Figure 6B). On

the other hand, bHLH11 physically interacts with and inhibits bHLH IVc TFs (Figure 5A, B). It is noteworthy the bHLH Ib genes were upregulated in both *bhlh11* mutants and *bHLH11-OX* plants. We hypothesize that a feedback regulation loop exists in the *bHLH11-OX* plants in which the Fe deficiency status caused by *bHLH11* overexpression promotes the upregulation of bHLH Ib genes. Similar feedback regulations are universal in Fe homeostasis. For example, loss-of-function mutations in the Fe transport-associated genes *IRT1*, *FRD3*, or *OPT3* lead to disruption of Fe homeostasis and cause the upregulation of bHLH Ib genes in Arabidopsis (Wang et al. 2007). Therefore, it is likely that the downregulation of *IRT1* and the low Fe status in the *bHLH11-OX* caused the upregulation of bHLH Ib genes by a feedback regulation. In support of this hypothesis, the expression levels of bHLH Ib genes were downregulated in the *pER8-bHLH11* plants after transient treatment with estradiol (Figure 5C), which ruled out the secondary effect caused by constitutive *bHLH11* overexpression.

Therefore, the expression levels of bHLH Ib genes are balanced by both bHLH IVc TFs and bHLH11. This study expands our knowledge of the Fe homeostasis transcription network mediated by bHLH Ib and IVc proteins. Based on our findings, we propose a putative working model for bHLH11 (Figure 7). bHLH11 transcript and protein abundance decrease with a decrease in the environmental Fe concentration. bHLH11 functions as an active repressor by recruiting TPL/TPR corepressors. bHLH11 interacts with and inhibits the transcriptional activation ability of bHLH IVc TFs. When Fe is sufficient or excessive, *bHLH11* transcription is activated and bHLH11 protein accumulates. Some of bHLH11 proteins are recruited by bHLH IVc TFs into the nucleus, where bHLH11 represses the transcription of Fe deficiency-responsive genes by interacting with TPL/TPR corepressors. This enables roots to control Fe uptake and avoid Fe toxicity. Under Fe-deficient conditions, *bHLH11* transcription slows down and bHLH11 protein degradation accelerates, which relieves the repression by bHLH11 to Fe

deficiency-responsive genes and facilitates Fe uptake. Our study provides experimental support for the existence of an elaborate system that allows plants to respond dynamically to Fe status. This mechanism is based on an equilibrium between the activation of Fe uptake-associated genes by bHLH IVc and their repression by bHLH11. Disruption of this equilibrium by misexpression of *bHLH11* disturbs Fe homeostasis. Therefore, bHLH11 is a key component of the Fe homeostasis signaling pathway.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* ecotype Columbia-0 was used as the wild type in this study. Plants were grown in long photoperiods (16-hour light/8-hour dark) or short photoperiods (10-hour light/14-hour dark) at 22°C. Surface sterilized seeds were stratified at 4°C for 2 d before being planted on media. Half Murashige and Skoog (MS) media with 1% sucrose, 0.8% agar A and the indicated FeEDTA concentration were used. Fe0 (0 µM Fe), Fe50 (50 µM Fe), Fe100 (100 µM Fe) and Fe300 (300 µM Fe).

Generation of CRISPR/Cas9-edited *bHLH11*

For CRISPR/Cas9-mediated editing of *bHLH11*, two target sites were designed by CRISPR-GE (Xie et al. 2017) to target the third and fourth exon of bHLH11, which were driven by the AtU3b promoter and respectively cloned into the pMH-SA binary vector carrying the Cas9 (Liang et al. 2016). The wild type plants were transformed and positive transgenic plants were selected on half-strength MS medium containing 20 µg/mL hygromycin. The positive transformants were sequenced and the homozygous mutants without hygromycin resistance were selected for further analysis.

442 **Generation of transgenic Plants**

443 HA-tag or VP16 domain were fused in frame with the full-length coding
444 sequence of *bHLH11* to generate *35S:HA-bHLH11* and *35S:bHLH11dm-VP16*
445 in the pOCA30 binary vector. HA-tagged bHLH11 was cloned into pER8 vector
446 (Zuo et al. 2001). These constructs were introduced into Arabidopsis plants
447 using the agrobacterium-mediated floral dip method.

448

449 **Yeast-two-hybrid assays**

450 Full-length bHLH11 was cloned into pGBKT7 as a bait to screen an
451 iron-depleted cDNA library. The full-length of bHLH IVc in the pGADT7 was
452 described previously (Li et al. 2016). Growth was determined as described in
453 the Yeast Two-Hybrid System User Manual (Clontech).

454

455 **Subcellular localization**

456 For the construction of *35S:bHLH11-mCherry*, mCherry-tag was fused with
457 bHLH11. *35S:bHLH34-GFP*, *35S:bHLH104-GFP*, *35S:bHLH105-GFP*,
458 *35S:bHLH115-GFP*, and *35S:GFP* were described previously (Lei et al. 2020).
459 *35S:bHLH11-mCherry* was co-expressed with various GFP-containing vectors
460 in tobacco cells. Epidermal cells were recorded on an OLYMPUS confocal
461 microscope. Excitation laser wave lengths of 488 nm and 563 nm were used
462 for imaging GFP and mCherry signals, respectively.

463

464 **Fluorescence complementation Assays**

465 The tripartite split-GFP fluorescence complementation assay was described as
466 previously (Liu et al. 2018; Lei et al. 2020). bHLH11 was fused with a
467 C-terminal GFP11 tag. The bHLH IVc genes were fused with an N-terminal
468 GFP10 tag. All vectors were introduced into *A. tumefaciens* (strain EHA105)
469 and the various combinations of Agrobacterial cells were infiltrated into leaves
470 of *Nicotiana benthamiana* by an infiltration buffer (0.2 mM acetosyringone, 10
471 mM MgCl₂, and 10 mM MES, PH 5.6). Gene expression was induced 1 day

472 after agroinfiltration by injecting 20 μ M β -estradiol in the abaxial side of the
473 leaves. Epidermal cells were recorded on a Carl Zeiss Microscopy.

474

475 **Co-immunoprecipitation Assay**

476 HA-bHLH11 and MYC-bHLH IVc or MYC-GFP were transiently expressed in
477 the *Nicotiana benthamiana* leaves and the leaves were infiltrated with MG132
478 12 hours before harvesting. 2 g leaf samples were used for protein extraction
479 in 2 ml IP buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM MgCl₂, 20%
480 glycerol, 0.2% NP-40, 1 X protease inhibitor cocktail and 1 X phosphatase
481 inhibitor cocktail from Roche). Lysates were clarified by centrifugation at 20,
482 000 g for 15 min at 4 °C and were immunoprecipitated using MYC antibody. IP
483 proteins were analyzed by immunoblot using anti-HA and anti-MYC antibody
484 respectively (Affinity Biosciences).

485

486 **Gene Expression Analysis**

487 Total root RNA was extracted by the use of the Trizol reagent (Invitrogen). For
488 the reverse transcription reaction, 1 μ g total RNA was used for cDNA synthesis
489 by oligo(dT)18 primer according to the manufacturer's protocol (Takara). The
490 resulting cDNA was subjected to relative quantitative PCR using the ChamQ™
491 SYBR qPCR Master Mix (Vazyme Biotech Co.,Ltd) on a Roche LightCycler
492 480 real-time PCR machine, according to the manufacturer's instructions. For
493 gene expression analysis in Arabidopsis plants, *ACT2* was used as an internal
494 control and gene copy number was normalized to that of *ACT2*. For gene
495 expression analysis in tobacco transient expression assays, *NPTII* was used
496 as an internal control and gene copy number was normalized to that of *NPTII*.
497 For the quantification of each gene, three biological replicates were used. The
498 quantitative reverse transcription-PCR primers were listed in Table S1.

499

500 **Fe Concentration Measurement**

501 To determine Fe concentration, rosette leaves from three-week-old seedlings

grown in soil were harvested and dried at 65 °C for 3 days. For each sample, about 100 mg dry weight was wet-ashed with 1.5 ml of 13.4 M HNO₃ and 1.5 ml of 8.8 M H₂O₂ for 20min at 220°C. Metal concentration was measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Three biological replicates were used for Fe concentration analysis.

Transient expression assays in Arabidopsis protoplasts

Arabidopsis mesophyll protoplasts preparation and subsequent transfection were performed as described previously (Wu et al. 2009). The promoter sequence of *bHLH38* was amplified from genomic DNA and cloned into pGreenII 0800-LUC vector. The coding sequences of GFP and various kinds of bHLHs (bHLH34, bHLH104, bHLH105, bHLH115, bHLH11, bHLH11dm and bHLH11dm-VP16) were respectively cloned into the pGreenII 62-SK vector under control of 35S promoter. For the reporter and effectors, 10 µg plasmid for each construct was used. After protoplasts preparation and subsequent transfection, firefly luciferase (LUC) and renillia luciferase (REN) activities were measured using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instructions. Relative (LUC) activity was calculated by normalizing against the REN activity.

Transient expression assays in Tobacco

Agrobacterium tumefaciens strains EHA105 was used in the transient expression experiments in tobacco. *pGAL4* promoter and BD domain were described previously (Li et al. 2016). *pGAL4* promoter was fused with NLS-GFP and cloned into the pOCA28 binary vector. 35S:BD, 35S:BD-bHLH104, 35S:BD-bHLH105 and 35S:HA-bHLH11 were constructed in the pOCA30 binary vector. For co-infiltration, different agrobacterium strains carrying different constructs were mixed prior to infiltration. Leaf infiltration was conducted in 3-week-old *N. benthamiana*. *NPTII* gene in the pOCA28 vector was used as the internal control. *GFP* transcript abundance was normalized to

532 that of *NPTII*.

533

534 **Immunoblotting**

535 For total protein extraction, roots were ground to a fine powder in liquid
536 nitrogen and then resuspended and extracted in RIPA buffer (50 mM Tris, 150
537 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 x
538 protease inhibitor cocktail [pH 8.0]). Isolation of cytoplasmic and nuclear
539 proteins was performed as described previously (Li et al. 2018). Sample was
540 loaded onto 12% SDS-PAGE gels and transferred to nitrocellulose membranes.
541 The membrane was blocked with TBST (10 mM Tris-Cl, 150 mM NaCl, and
542 0.05% Tween 20, pH 8.0) containing 5% nonfat milk (TBSTM) at room
543 temperature for 60 min and incubated with primary antibody in TBSTM
544 (overnight at 4°C). Membranes were washed with TBST (three times for 5 min
545 each) and then incubated with the appropriate horseradish
546 peroxidase-conjugated secondary antibodies in TBSTM at room temperature
547 for 1.5 h. After washing three times, bound antibodies were visualized with
548 ECL substrate.

549

550 **Supplemental data**

551 **Supplemental Figure S1.** Phenotypes of *bHLH11-OX* plants.

552 **Supplemental Figure S2.** Genotypes of *bhlh11* mutants.

553 **Supplemental Figure S3.** Prediction of NLS in bHLH11.

554 **Supplemental Figure S4.** Phenotypes of *pER8-bHLH11* transgenic plants.

555 **Supplemental Figure S5.** Expression of *IRT1* and *FRO2* in *bHLH11dm-VP16*
556 transgenic plants.

557 **Supplemental Figure S6.** Conserved EAR motif in the bHLH11 homologs
558 from various plants species.

559 **Supplemental Table S1.** Primers used in this paper.

560

561

562

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568

569 **AUTHOR CONTRIBUTIONS**

570 G.L. conceived the project. Y.L., R.L., M.P., C.L., Z. L., and G.L. constructed
571 plasmids, M.P. and Y.C generated transgenic plants, and Y.L. and R.L.
572 characterized plants, determined gene and protein expression and conducted
573 cellular assays. Y.L. and G.L. wrote the manuscript and all authors discussed
574 and approved the manuscript.

575

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744

Iron responsive genes in WT, *bHLH11-OX* plants and *bhlh11* mutants.

Fe100			Fe0				
7-24	<i>bhlh11-1</i>	<i>bhlh11-2</i>	WT	<i>OX-20</i>	<i>OX-24</i>	<i>bhlh11-1</i>	<i>bhlh11-2</i>
19±0.02 ^b	1.82±0.22 ^c	1.65±0.17 ^c	59.72±2.61 ^A	0.33±0.07 ^A	0.38±0.04 ^A	75.09±2.64 ^C	70.21±1.30 ^C
12±0.04 ^b	1.29±0.05 ^c	1.51±0.15 ^c	69.28±1.80 ^C	0.49±0.04 ^A	0.52±0.05 ^A	98.37±3.64 ^C	97.06±8.66 ^C
17±0.25 ^c	3.48±0.23 ^b	3.62±0.13 ^b	80.79±2.32 ^A	106.52±4.12 ^B	94.5±4.63 ^B	96.04±1.82 ^B	97.28±3.71 ^B
13±0.57 ^c	2.62±0.57 ^b	3.00±0.14 ^b	43.06±3.33 ^A	61.67±2.36 ^B	61.28±0.82 ^B	64.08±2.56 ^B	62.48±4.31 ^B
19±0.92 ^c	2.04±0.15 ^b	2.31±0.24 ^b	72.87±2.72 ^A	105.75±3.00 ^B	101.34±0.89 ^B	102.64±2.38 ^B	97.54±2.48 ^B
17±0.49 ^b	1.33±0.23 ^a	1.35±0.25 ^a	41.01±1.94 ^A	58.65±3.83 ^B	68.15±1.67 ^C	65.09±4.20 ^{BC}	67.5±2.98 ^C
7±0.20 ^a	1.05±0.08 ^a	1.04±0.05 ^a	3.12±0.12 ^A	3.21±0.11 ^A	3.10±0.14 ^A	3.07±0.24 ^A	3.00±0.11 ^A
15±0.24 ^c	2.64±0.49 ^b	3.48±0.63 ^{bc}	5.29±0.30 ^A	7.00±0.13 ^{BC}	7.82±0.41 ^C	6.21±0.10 ^B	6.59±0.63 ^B
10±0.19 ^c	1.53±0.22 ^b	1.30±0.23 ^{ab}	3.34±0.28 ^A	4.18±0.15 ^B	4.56±0.46 ^B	4.55±0.43 ^B	5.04±0.25 ^B
15±0.30 ^b	1.52±0.19 ^b	1.47±0.22 ^b	5.65±1.00 ^C	61.03±6.82 ^D	69.3±5.64 ^D	8.43±0.55 ^C	9.5±1.46 ^C
19±0.16 ^a	1.05±0.07 ^a	1.22±0.17 ^a	1.97±0.18 ^A	2.12±0.20 ^A	2.02±0.12 ^A	2.20±0.21 ^A	2.17±0.29 ^A
10±0.41 ^a	1.16±0.26 ^a	1.29±0.37 ^a	5.41±0.57 ^{AB}	5.80±1.32 ^B	6.00±1.75 ^B	4.96±0.29 ^A	6.26±0.71 ^B
5±0.25 ^a	0.95±0.18 ^a	0.99±0.29 ^a	2.91±0.14 ^A	4.01±0.40 ^B	3.23±0.28 ^B	3.89±0.21 ^B	3.85±0.27 ^B
3.06±10.71 ^c	1.07±0.06 ^a	1.10±0.09 ^b	0.52±0.04 ^B	111.79±8.64 ^C	125.71±7.98 ^C	0.49±0.02 ^A	0.54±0.07 ^A

Fe0 media for 7d. RNA was prepared from root tissues. The expression of *ACT2* was used to normalize mRNA levels, and the gene 100 was set to 1^a. Different letters above each bar indicate statistically significant differences as determined by one-way ANOVA comparison test (P < 0.05) and .

Figure Legends

Figure 1. *bhlh11* loss-of-function mutants are sensitive to excessive Fe.

(A) Phenotypes of *bhlh11* mutants. Two-week-old seedlings grown on Fe0, Fe100 or Fe300 media.

(B) Shoot biomass of *bhlh11* mutants. Fresh weight of two-week-old shoots grown on Fe0, Fe100 or Fe300 media. Student' t test indicated that the values marked by an asterisk are significantly different from the corresponding wild-type value ($P < 0.05$).

(C) Fe concentration of rosette leaves of 3-week-old WT and *bhlh11* plants grown in soil. Student' t test indicated that the values marked by an asterisk are significantly different from the corresponding wild-type value ($P < 0.05$).

Figure 2. Response of bHLH11 to Fe status

(A) RT-qPCR analysis of *bHLH11* expression. Four-day-old plants grown on Fe100 media were shifted to Fe0, Fe50, Fe100, and F300 media for 3 days. Roots were used for RNA extraction and RT-qPCR. The different letters above each bar indicate statistically significant differences as determined by one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$).

(B) Degradation of bHLH11 in response to Fe deficiency. Seven-day-old WT and *bHLH11-OX-20* seedlings grown on Fe100 media were transferred to Fe0 or Fe300 media, and root samples were harvested after 1, 2, and 3 days. anti-HA was used to detect HA-bHLH11. β -tubulin was detected by anti- β -tubulin and used as a loading control.

(C) Subcellular localization of bHLH11. The free mCherry, bHLH11-mCherry or bHLH11-NLS-mCherry were transiently expressed in tobacco leaves.

Figure 3. bHLH11 physically interacts with bHLH IVc TFs.

(A) Yeast two-hybrid analysis of the interaction between bHLH11 and bHLH IVc TFs. Yeast cotransformed with different BD and AD plasmid combinations

was spotted on synthetic dropout medium lacking Leu/Trp (SD–W/L) or Trp/Leu/His/Ade (SD –W/L/H/A).

(B) Interaction of bHLH11 and bHLH IVc TFs in plant cells. Tripartite split-sfGFP complementation assays were performed. bHLH34, bHLH104, bHLH105, and bHLH115 were fused with GFP10, and bHLH11 was fused with GFP11. The combinations indicated were introduced into *Agrobacterium* and co-expressed in *Nicotiana benthamiana* leaves..

(C) Co-IP analysis of the interaction between bHLH11 and bHLH IVc TFs. Total proteins from different combinations of HA-bHLH11 and MYC-GFP, MYC-bHLH34, MYC-bHLH104, MYC-bHLH105, or MYC-bHLH115 were immunoprecipitated with anti-MYC followed by immunoblotting with the indicated antibodies. MYC-GFP was used as the negative control. Protein molecular weight (in kD) is indicated to the left of the immunoblot.

Figure 4 Change of bHLH11 subcellular localization.

(A) Location of bHLH11 in the absence or presence of bHLH IVc. bHLH11-mCherry was co-expressed with bHLH IVc TFs. The combination of bHLH11-GFP and free mCherry was used as a negative control. Transient expression assays were performed in tobacco leaves.

(B) Immunoblot analysis of bHLH11 protein distributions in the cytoplasm and nuclear fractions. Seven-day-old *bHLH11-OX-20* seedlings grown on Fe100 media were transferred to Fe0 or Fe300 media. Root samples were harvested after 3 days, and cytoplasmic and nuclear proteins were extracted and subjected to immunoblot analysis with the indicated antibodies.

Figure 5. bHLH11 antagonizes the transcriptional activation ability of bHLH IVc TFs.

(A) bHLH11 represses the functions of bHLH IVc TFs. Schematic diagram of the constructs transiently expressed in *Arabidopsis* protoplasts. The LUC/REN ratio represents the LUC activity relative to the internal control (REN driven by

the 35S promoter). The asterisk indicates a significant difference as determined by Students' t test.

(B) bHLH11 inhibits the functions of bHLH IVc TFs by direct protein–protein interaction. The schematic diagram shows the constructs used in the transient expression assays in tobacco leaves. The abundance of *GFP* was normalized to that of *NPTII*. The asterisk indicates a significant difference as determined by Students' t test.

(C) Expression of bHLH Ib genes in *pER8-bHLH11* plants. Seven-day-old plants grown on Fe0 media were transferred to Fe0 media with or without 4 μ M estradiol for 6 h, and root samples were harvested and used for RNA extraction and RT-qPCR. The different letters above each bar indicate statistically significant differences as determined by one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$).

Figure 6. bHLH11 acts as a repressor by recruiting TPL/TPR corepressors.

(A) Schematic diagram of the various mutated versions of bHLH11. The mutated amino acid is indicated in red. bHLH11m1, the first EAR mutated. bHLH11m2, the second EAR mutated. bHLH11dm, both double EARs mutated. bHLH11dm-VP16, bHLH11dm fused with the VP16 domain.

(B) The EAR motifs are required for the interaction between bHLH11 and TPL/TPR. Yeast cotransformed with different BD and AD plasmid combinations was spotted on synthetic dropout medium lacking Leu/Trp (SD –T/L) or Trp/Leu/His/Ade (SD –T/L/H/A).

(C) The schematic diagram shows the constructs used in the transient expression assays in (D).

(D) The EAR motifs are required for the repression of bHLH11. Arabidopsis protoplasts were used for transient expression assays.

(E) Phenotypes of *bHLH11dm-VP16* and *bHLH11-OX* plants. Seven-day-old seedlings grown on Fe0 or Fe100 media are shown.

Figure 7. A working model of bHLH11 in Fe homeostasis.

bHLH11 functions as an active repressor by recruiting TPL/TPR corepressors. Under Fe-sufficient conditions, *bHLH11* message is activated and its protein accumulates. bHLH11 inhibits the transactivity of bHLH IVc TFs to bHLH Ib genes. bHLH11 may directly regulate the expression of Fe deficiency-responsive gene *IRT1* and *FRO2* (as indicated by a question sign). The repressor function of bHLH11 allows plants to avoid Fe toxicity. Under Fe-deficient conditions, unknown proteins repress the transcription of *bHLH11*, which alleviates the bHLH11-mediated repression to bHLH IVc TFs. bHLH IVc TFs promotes the transcription of bHLH Ib genes. bHLH Ib TFs and FIT activate the expression of the Fe uptake-associated gene *IRT1* and *FRO2*. The question signs indicate unknown proteins.

A **B** **C**

A **B** **C**

A **C** **B**

A **B**

A **B** **C**

+Fe

-Fe