1 **Arabidopsis HB52 mediates the crosstalk between ethylene and auxin**
2 **signaling pathways by regulating *PIN2*, *WAG1*, and *WAG2* during**
3 **primary root elongation**

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21 **Abstract**

22 The gaseous hormone ethylene participates in many physiological processes of plants.
 23 It is well known that ethylene-inhibited root elongation involves basipetal auxin
 24 delivery requiring PIN2. However, the molecular mechanism how ethylene regulates
 25 *PIN2* is not well understood. Here, we report that the ethylene-responsive HD-Zip
 26 gene *HB52* is involved in ethylene-mediated inhibition of primary root elongation.
 27 Using biochemical and genetic analyses, we demonstrated that *HB52* is
 28 ethylene-responsive and acts immediately downstream of EIN3. *HB52* knock-down
 29 mutants are insensitive to ethylene in primary root elongation while the
 30 overexpression lines have dramatically shortened roots like ethylene treated plants.
 31 Moreover, *HB52* upregulates *PIN2*, *WAG1*, and *WAG2* by directly binding to their
 32 promoter, leading to an enhanced basipetal auxin delivery to the elongation zone and
 33 thus inhibiting root growth. Our work uncovers *HB52* as an important crosstalk node
 34 between ethylene signaling and auxin transport in root elongation.

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36

37 Introduction

38 Ethylene is a gaseous phytohormone which regulates a multitude of processes at
39 trace levels. It is well known for triggering the shedding of leaves, the ripening of
40 fruits, and the defense of plants. It also plays an indispensable role in root
41 development (Alonso and Ecker, 1999; Grbić and Bleecker, 2003; Chaves and
42 Mello-Farias, 2006; Ruzicka et al., 2007). Exogenous treatment with ethylene (C₂H₄)
43 or its biosynthesis precursor 1-aminocyclopropane-1-carboxylic acid (ACC) leads to
44 the inhibition of primary root elongation, the increase of primary root width, and the
45 induction of ectopic root hairs (Masucci and Schiefelbein, 1996; Smalle and Van Der
46 Straeten, 1997; Le et al., 2001). These three ethylene induced responses will promote
47 soil penetration and greater anchorage on the ground.

48 Great advances in ethylene signaling pathway have been made in the past decade
49 using genetic approaches in Arabidopsis (Merchante et al., 2013). In the absence of
50 ethylene, the receptors and other related proteins recruit the Raf-like kinase CTR1
51 which phosphorylates the C-terminal end of EIN2, thus preventing it from
52 translocating into the nucleus to stabilize the downstream transcription factors
53 EIN3/EIL1. In the presence of ethylene, the hormone binds to the receptors thus
54 inactivating CTR1, so the unphosphorylated C-terminal end of EIN2 can be cleaved
55 and moves into the nucleus to stabilize EIN3/ EIL1 which will activate the
56 downstream transcriptional cascade (Gao et al., 2003; Ju et al., 2012; Qiao et al., 2012;
57 Wen et al., 2012).

58 Intriguingly, mutants of auxin synthesis, signaling pathway or transport show
59 aberrant responses to ethylene, indicating crosstalk between these two hormones. For
60 example, mutations in auxin biosynthesis genes such as *ASA1*, *ASB1*, *TAR1* and *TAA1*
61 exhibit ethylene-insensitive root phenotypes (Stepanova et al., 2005; Stepanova et al.,
62 2008). *YUC* genes also play key roles in ethylene-mediated root response (Won et al.,
63 2011). Mutants of *AXR2/IAA7* and *AXR3/IAA17* which encode transcription regulators
64 in the auxin signal pathway exhibit insensitive root growth to ethylene (Alonso et al.,
65 2003). PIN2 and AUX1, two of the auxin transport components are also involved in

66 ethylene-mediated root response (Ruzicka et al., 2007).

67 Plants have a considerable number of transcription factors which play vital roles
68 in the different development process. Among all the families of transcription factors,
69 the HD-Zip family is unique to plant. These proteins display a singular combination
70 of a homeodomain with a leucine zipper working as a dimerization motif. This family
71 consists of 47 members and can be classified into four subfamilies (Ariel et al., 2007).
72 *ATHB1* participates in the determination of leaf cell fate, whereas *ATHB13* and
73 *ATHB23* are involved in cotyledon and leaf development (Aoyama et al., 1995;
74 Nakamura et al., 2006). *HAT2* overexpression lines have a representative phenotype
75 of auxin-overproducing mutants indicating a role in auxin-mediated development
76 (Delarue et al., 1998; Sawa et al., 2002). *PHV*, *PHB*, and *REV* have similar functions
77 during embryogenesis and leaf polarity determination (Prigge and Clark, 2006).
78 *ATHB10*, *ATML1*, and *PDF2* play important roles in cell fates establishment by
79 regulating cell layer-specific gene expression (Abe et al., 2003). Although some
80 proteins in this family have been studied well in the past few years, others still need
81 further investigation.

82 In this study, we report an HD-Zip gene *HB52* which is involved in
83 ethylene-mediated primary root elongation. *HB52* knock-down mutants are
84 insensitive to ethylene in primary root elongation while *HB52* overexpression lines
85 have shortened roots similar to ethylene treated plants. Biochemical and genetic
86 assays showed that *HB52* is a direct target of EIN3. *DR5::GUS* in *HB52* mutants
87 showed altered auxin basipetal transport. Further analyses demonstrated that *HB52*
88 could directly regulate *PIN2*, *WAG1*, and *WAG2*. Moreover, a clear PIN1 and PIN3
89 apical polarity in the stele and PIN2 apical polarity in the cortex were observed in the
90 *HB52* overexpression line. Our results indicate that *HB52* plays a vital role in the
91 inhibition of ethylene-induced primary root growth in Arabidopsis and acts as the
92 crosstalk node between ethylene and auxin signaling pathways in primary root
93 elongation.

94

Results

Expression pattern and subcellular localization of HB52

To investigate the expression pattern of *HB52*, we detected the transcription level of *HB52* in different tissues of 4-week old plants by quantitative RT-PCR. The strongest expression was observed in roots followed by stem and rosette leaves (Figure 1A). To further confirm this result, we generated *HB52pro:GUS* transgenic lines. Histochemical analysis of the transgenic lines showed that *HB52* promoter-driven GUS reporter was primarily expressed in the root tip and hypocotyl base of 4-day old young seedlings (Figure 1B, C and D). In 10-day old seedlings, GUS staining was mainly observed in roots and petiole of rosette leaves (Figure 1E). In mature plants, GUS staining was only found in roots (Figure 1F).

To investigate the subcellular localization of *HB52*, we generated *35S:HB52-GFP* transgenic lines. Clear fluorescence was observed in the nucleus under confocal laser scanning microscope (Figure 1G). The nucleus localization of *HB52* is in coincidence with its function as a transcription factor.

HB52 is responsive to ethylene, which depends on ethylene signaling

To confirm whether *HB52* is regulated by ethylene and determine its position in ethylene signaling pathway, we detected the transcript level of *HB52* in the wild type (Col-0) and ethylene signaling mutants using quantitative RT-PCR. *HB52* was upregulated by exogenous ACC (Figure 2A). Moreover, *HB52* was down regulated in ethylene signaling-blocked mutants *ein2-5* and *ein3-leil1* and upregulated in ethylene signaling-enhanced mutants *35S:EIN3-GFP* and *ctr1-1* without or with exogenous ACC (Figure 2A). To confirm this, we introduced *HB52pro:GUS* into *ein2-5*, *ein3-leil1*, *35S:EIN3-GFP* and *ctr1-1* background, respectively. The GUS staining of *HB52pro:GUS* was lighter in *ein2-5* and *ein3-leil1* background while darker in *35S:EIN3-GFP* and *ctr1-1* background when compared with *HB52pro:GUS* without or with exogenous ACC (Figure 2B and 2C). These results indicate that *HB52* acts downstream of *EIN3* and *EIL1*.

124

125 ***HB52* regulates primary root elongation in response to ethylene**

126 To study the role of *HB52* in root elongation in response to ethylene, we obtained
 127 the mutant CS909234 with a T-DNA insertion in the promoter of *HB52* from ABRC
 128 (Figure S1) and generated an estradiol-induced RNAi line RNAi-6. For clarity, the
 129 mutant CS909234 is renamed *hb52*. The transcript level of *HB52* in *hb52* and RNAi-6
 130 was significantly reduced compared with that of the wild type (Figure 3A).
 131 Meanwhile, we tried to generate *HB52* overexpression lines driven by 35S promoter
 132 but the transgenic plants failed to set seeds due to aberrant development of flowers
 133 (data not shown). Therefore, we generated *HB52* overexpression lines driven by
 134 estradiol-inducible promoter instead. The transcript level of *HB52* in three
 135 representative overexpression lines OX11-5, OX35-2, OX14-1 increased 30, 250 and
 136 870 fold, respectively (Figure 3A). The relative primary root elongation of the three
 137 overexpression lines decreased to 87%, 66%, 43% of the wild type after induction
 138 (Figure S2B, left panel). Clearly, the primary root length is negatively correlated with
 139 *HB52* expression level.

140 If germinated on MS medium with estradiol directly, the overexpression lines
 141 OX14-1 exhibited yellow colored cotyledon that might be caused by the high
 142 expression level of *HB52* (Figure S2A). To test the response of *HB52* mutants to
 143 ethylene and avoid the influence of yellow colored cotyledon, we germinated the
 144 seeds on MS medium for 3 days and then transferred the seedlings to MS medium
 145 with estradiol for another 3 days to induce gene expression. Afterwards, the seedlings
 146 were transferred to MS medium with estradiol supplemented with different
 147 concentration of ACC for 4 days to measure primary root elongation. Under 0 μ M
 148 ACC, the primary root elongation of the knock-down mutants (RNAi-6 and *hb52*)
 149 was comparable to that of the wild type control while it was significantly reduced in
 150 the overexpression lines, among which the primary root elongation was negatively
 151 correlated with *HB52* expression levels (Figure 3B, top panel). In response to ACC,
 152 the two *HB52* knock-down lines and three overexpression lines were all less sensitive
 153 in root elongation compared with Col-0 (Figure 3B and C). Among the three

overexpression lines, OX14-1 is the least sensitive line to ACC in root elongation followed by OX35-2 (Figure 3C). These results indicate that HB52 plays an important role in ethylene-inhibited primary root elongation.

In addition to altered primary root elongation, we observed other root phenotypes associated with varied *HB52* expression levels, which include collapsed root meristem of the overexpression lines (Figure S2A) and altered root gravitropic response of the knock-down mutants and overexpression lines (Figure S3).

***HB52* is a direct target of EIN3**

We have previously shown that *HB52* acts downstream of *EIN3* and *EIL1*. So we next explored whether *HB52* is a direct target of EIN3 and EIL1. Three putative EIN3-binding sites (EBS, TACAT or TTCAAA) were found in the promoter of *HB52* (Konishi and Yanagisawa, 2008; Zhong et al., 2009; An et al., 2012; Li et al., 2013) (Figure 4A). We performed chromatin immunoprecipitation (ChIP) assays using *35S:EIN3-GFP* and *35S:EIL1-GFP* transgenic plants. Marked enrichment of the region containing cis2 site (TACAT) was detected in *35S:EIN3-GFP* transgenic plants by ChIP-PCR assays (Figure 4B and 4C), indicating that EIN3 binds to this region *in vivo*. Furthermore, we conducted yeast-one-hybrid to determine whether EIN3 and EIL1 could directly bind to the EBS in the promoter of *HB52*. The result showed that EIN3 was able to bind to the cis2 site in the promoter of *HB52* (Figure 4D). Taken together, these data suggest that *HB52* is a direct target of EIN3.

To further confirm that *HB52* acts downstream of *EIN3*, we crossed *hb52* with *35S:EIN3-GFP* and *ctr1-1* separately. *ctr1-1hb52* had the same point mutation with *ctr1-1* and *35S:EIN3-GFP* had the same expression level of *EIN3* with *35S:EIN3-GFP* (Figure S4A and S4B). *HB52* expression level decreased in *ctr1-1hb52* and *35S:EIN3-GFP* (Figure S4C). The genetic assays showed that the roots of *35S:EIN3-GFP* and *ctr1-1hb52* are longer than that of *35S:EIN3-GFP* and *ctr1-1* without and with exogenous ACC (Figure 5A and 5B). This genetic evidence strongly supports that *HB52* acts downstream of *EIN3*.

184 **HB52 directly regulates *PIN2*, *WAG1*, and *WAG2***

185 We have noticed that *HB52* knock-down lines and overexpression lines were all
186 insensitive to ACC in root elongation. Obviously, *HB52* plays an important role in
187 ethylene-mediated root elongation. However, the underlying molecular mechanism is
188 unknown. We introduced *DR5-GUS* reporter into *hb52* and OX35-2 background by
189 crossing to see if there is any change of auxin level in root tip. Both lines were
190 confirmed by detecting the transcript level of *HB52* (Figure 6A). Exogenous ACC
191 clearly induces the expression of the *DR5:GUS* reporter in the elongation zone of the
192 wild type but not in the *hb52* background, indicating a blockage in auxin basipetal
193 transport, while the expression of *DR5:GUS* is significantly reduced in the OX35-2
194 background without or with exogenous ACC (Figure 6B). Taken together, these
195 results suggest that the basipetal transport of auxin is altered by HB52.

196 To investigate the role of HB52 in auxin basipetal transport, we examined the
197 transcript level of *PID*, *WAG1*, *WAG2* and other auxin transport related genes. As
198 shown in Figure 6C, *PIN2*, *WAG1*, and *WAG2* were downregulated in *hb52* and
199 upregulated in OX14-1. Moreover, several HB52 binding sites were found in
200 promoters of *PIN2*, *WAG1*, and *WAG2*, suggesting that these three genes are direct
201 targets of HB52.

202 To confirm that *PIN2*, *WAG1*, and *WAG2* are direct targets of HB52, we
203 demonstrated that HB52 was able to directly bind to at least one homeodomain
204 binding site in the promoter of these three genes by using ChIP-PCR,
205 yeast-one-hybrid, and EMSA (Figure 7, 8 and 9).

206 In order to confirm genetically that *PIN2*, *WAG1*, and *WAG2* act downstream of
207 HB52, we crossed the knockout mutants of *PIN2* (*pin2*, CS8058), *WAG1* (*wag1*,
208 Salk_002056) and *WAG2* (*wag2*, Salk_070240) with the *HB52* overexpression line
209 (OX35-2), respectively and confirmed the expression of *HB52* (Figure 10A). The
210 results in Figure 10B and 10C show that the primary roots of these hybrid lines are
211 longer than the *HB52* overexpression line with different degrees as predicted. These
212 results suggest that HB52 depends on *WAG1*, *WAG2*, and *PIN2* for its function in
213 ethylene-mediated root elongation.

214

215 **Discussion**

216 Synergistic effects of auxin and ethylene have been extensively studied in the
217 regulation of root elongation. Ethylene has been shown to increase auxin synthesis,
218 auxin transport to the elongation zone, and auxin signaling at the root tip (Pickett et
219 al., 1990; Alonso et al., 2003; Stepanova et al., 2005; Ruzicka et al., 2007; Swarup et
220 al., 2007; Stepanova et al., 2008; Mao et al., 2016). The HD-Zip transcription factors
221 are a unique family in plants and divided into 4 subfamilies I-IV mainly based on
222 their structure and function. *HB52* belongs to HD-ZIP I and has not been revealed for
223 its role in plants. Members of this subfamily have been shown to be involved in
224 abiotic stress response, ABA-mediated regulation, de-etiolation, and blue-light
225 signaling (Ariel et al., 2007). In this study, we identified that ethylene-responsive
226 *HB52* acts directly downstream of EIN3 to affect auxin basipetal transport by
227 regulating *WAG1*, *WAG2*, and *PIN2*.

228 It is known that *HB52* can be upregulated by ethylene in the root in public data
229 such as e-FP browser. A previous study also shows that EIN3, a master regulator of
230 the ethylene signaling pathway, binds directly to the promoter of *HB52* based on the
231 data of EIN3 ChIP-Seq experiments (Chang et al., 2013). So we speculate that it may
232 play a role in ethylene-mediated root regulation. To investigate its function, we first
233 obtained the *HB52* knock-down mutant and overexpression lines. Both *HB52*
234 knock-down mutant and overexpression lines are less sensitive to exogenous ACC in
235 root elongation than wild type (Figure 3). Moreover, the primary roots of
236 *35S:EIN3-GFP**hb52* and *ctr1-1 hb52* are longer than *35S:EIN3-GFP* and *ctr1-1*
237 respectively, which further supports the role of *HB52* in ethylene-mediated root
238 elongation (Figure 5). Both ChIP-PCR and yeast-one-hybrid experiments confirm that
239 EIN3 can bind to the promoter of *HB52* (Figure 4), consistent with EIN3 ChIP-Seq
240 data (Chang et al., 2013). The expression pattern of *HB52pro::GUS* reporter in
241 transgenic lines also matches the function of *HB52* in the root (Figure 1 and 2).

242 To investigate the specific mechanism by which *HB52* controls root elongation.

243 We introduced *DR5:GUS* reporter into *hb52* and OX35-2 background. When treated
 244 with ACC, the staining of *DR5:GUS* in *hb52* background showed a blockage in auxin
 245 basipetal transport (Figure 6B), which explains the insensitivity of knock-down lines
 246 to ethylene (Figure 3B and 3C). The staining of *DR5:GUS* is significantly reduced in
 247 OX35-2 background mainly due to the aberrant development of meristematic zone in
 248 the root (Figure 6B and S2A). This is the reason why overexpression lines are
 249 insensitive to ACC because ethylene-mediated root inhibition needs more auxin
 250 basipetal transport from the meristematic zone to the elongation zone (Ruzicka et al.,
 251 2007). The aberrant development of meristem is probably the cause of agravitropism
 252 (Figure S3) since auxin redistribution in the meristematic zone is of vital importance
 253 in regulating gravitropic response (Petrasek and Friml, 2009).

254 The root phenotype of *HB52* overexpression lines is very similar to that of *PID*,
 255 *WAG1* and *WAG2* overexpression lines. Estradiol-induced overexpression of *PID*,
 256 *WAG1* or *WAG2* led to reduced *DR5:GUS* expression, loss of gravitropism and
 257 collapse of root meristem. It was reported that the collapsed root meristem can be
 258 rescued by NPA (Benjamins et al., 2001; Dhonukshe et al., 2010). We previously
 259 obtained *35S: HB52* lines with severe fertility problems (data not shown) just like the
 260 *35S: PID* lines due to abnormal flower development (Benjamins et al., 2001). A
 261 frequent collapse of root meristem was observed in overexpression lines and can be
 262 rescued by NPA (Figure S2B, right panel). Considering the fact that auxin transport
 263 was altered in *HB52* mutants and the overexpression lines had so many similarities
 264 with AGC3 kinase overexpression lines (Figure 6B, S2 and S3), we detected the
 265 transcript level of the genes related to auxin transport and found *PIN2*, *WAG1*, and
 266 *WAG2* were downregulated in *HB52* knock-down mutants and upregulated in
 267 overexpression lines (Figure 6C).

268 It has been shown that *pin2/eir1* is insensitive to ethylene in root elongation and
 269 exogenous ACC upregulates the *PIN2* expression of *proPIN2:GUS* and
 270 *proPIN2:PIN2-GFP*, indicating *PIN2* is involved in the ethylene-mediated root
 271 inhibition. But *PIN2* is not a direct target of EIN3 (Benjamins et al., 2001; Chang et
 272 al., 2013). The link between ethylene and *PIN2* is still to be revealed. *PID*, *WAG1*,

and *WAG2* belong to the plant-specific AGCVIII family of kinases and work redundantly to instruct PIN apical polarity in root development. The most distal cells of the *pidwag1wag2* root epidermis displayed basal localization of PIN2 as compared with its apical localization in wild type, while overexpression of these three genes leads to apically localized PIN1 in the root stele, PIN2 in the cortex and PIN4 in the root meristem (Dhonukshe et al., 2010). It has been demonstrated that PIN2 in the epidermis is responsible for auxin basipetal transport and required for root gravitropic response (Ruzicka et al., 2007). The root of *HB52* knock-down mutant is agravitropic and show partly blocked auxin basipetal transport (Figure S3 and 6B) mainly due to the less apical localization of PIN2 in the epidermis caused by downregulation of *PIN2*, *WAG1*, and *WAG2* (Figure 6C). By using yeast-one-hybrid, ChIP-PCR, EMSA, and genetic analyses, we further proved that *PIN2*, *WAG1*, and *WAG2* are direct targets of HB52 in ethylene-mediated root inhibition (Figure 7, 8, 9 and 10).

Taken together, our results support a model where ethylene stabilizes EIN3 and upregulates *HB52*. *HB52* then increases the expression of *PIN2*, *WAG1*, and *WAG2*. As a result, more auxin is transported to the elongation zone, leading to inhibition of root elongation.

Materials and Methods

Plant materials and growth conditions.

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used as wild-type. A homozygous *HB52* knock-down mutant CS909234 was ordered from Arabidopsis Biological Resource Center. The OX11-5, 35-2, 14-1, 18-4, RNAi-6, *HB52pro::GUS*, *35S::HB52-GFP*, *35S::EIN3-GFP* transgenic plants were obtained by *Agrobacterium* (C58C1) -mediated transformation using the *Arabidopsis* floral-dip method. For OX11-5, 35-2, 14-1 and 18-4, the *HB52* coding sequence was amplified by pER8-HB52-P1 and pER8-HB52-P2 and cloned into pER8. For RNAi-6, about 200bp of the *HB52* coding sequence was amplified by RNAi-P1 and RNAi-P2 and then by

RNAi-P3 and RNAi-P4, both segments were cloned into phj33, and then shuttled it into the pER8. For *HB52pro:GUS*, the promoter of HB52 were amplified by GUS-HB52-P1 and GUS-HB52-P2 and cloned into pDONR207, and then shuttled it into the pCB308R. For *35S:HB52-GFP*, the HB52 coding sequence without a stop codon were amplified by GFP-HB52-P1 and GFP-HB52-P2 and cloned into pDONR207, and then shuttled it into the pGWB5.

Several plant materials were previously described: *ein2-5* (Alonso et al., 1999), *ein3-1 eil1-1* (Alonso et al., 2003), *ctr1-1* (Kieber et al., 1993), *35S:EIN3-GFP*, *HB52pro:GUSein2-5*, *HB52pro:GUSein3-1eil1*, *HB52pro:GUS35S:EIN3-GFP* and *HB52pro:GUSctr1-1* were crossed by *HB52pro:GUS* and *ein2-5*, *ein3-1eil1*, *35S:EIN3-GFP* and *ctr1-1* separately. *ctr1-1* CS909234 and *35S:EIN3-GFP* CS909234 were crossed by CS909234 with *ctr1-1* and *35S:EIN3-GFP* separately.

Arabidopsis seeds were surface sterilized in 10% bleach for 15 minutes and washed with distilled water for 6 times. Then the seeds were vernalized at 4°C for 3 days and vertically germinated on 1/2MS medium (Murashige and Skoog). If transferred to soil, all plants were grown under long day conditions (16-h light / 8-h dark) at 22–24°C.

319

320 **Histochemical GUS staining and fluorescence observation**

Histochemical GUS staining of transgenic plants was performed as previously described (Mao et al., 2016). Images were captured using an OLYMPUS IX81 microscope and HiROX (Japan) MX5040RZ.

Fluorescence observation of GFP transgenic plants was imaged using ZEISS710 confocal laser scanning microscope: 543nm for excitation and 620 nm for emission.

Fluorescence observation of Propidium iodide (PI) stained transgenic plants.

Seedlings were incubated in 10 mg/mL propidium iodide for 3 minutes and washed twice in water. The stained seedlings were imaged using ZEISS710 confocal laser scanning microscope: 488nm for excitation and 510 nm for emission.

330

331 **RT-PCR and quantitative RT-PCR analysis**

332 Total RNA was isolated using TRIzol reagent (Invitrogen) and reversed by
333 TransScript RT kit (Invitrogen). Then cDNA was used for RT-PCR and quantitative
334 RT-PCR. For RT-PCR analysis, the PCR products were amplified and examined on 2%
335 agarose gel. Quantitative RT-PCR was performed on StepOne real-time PCR system
336 using SYBR Premix Ex Taq II kit. Genes expression level was normalized by
337 Ubiquitin5 (UBQ5, At3g62250).

338

339 **Yeast-one-hybrid assay**

340 Yeast one-hybrid assay was carried out as described previously (Mao et al., 2016).
341 The coding sequence of proteins was cloned into pAD-GAL4-2.1 (AD vector) and the
342 putative protein binding sites were cloned into pHIS2 (BD vector).

343

344 **Starch granules staining**

345 Starch granule staining was performed as described previously (Sabatini et al.,
346 1999).

347

348 **ChIP assay**

349 ChIP assay was carried out as described previously (Cai et al., 2014).

350

351 **EMSA assay**

352 Competitors were commercially synthesized and free probes were synthesized
353 with biotin labelled at the 5' end. The coding sequence of HB52 was cloned into
354 pMAL-C2 and the HB52-MBP fusion protein was expressed in Rosseta2 strain.
355 EMSA assay was performed using LightShift™ EMSA Optimization and Control
356 Kit (20148×) according to the manufacturer's instructions.

357

358 **Supplemental information**

359 Figure S1. Identification of the T-DNA insertions in CS909234 (*hb52*).

360 Figure S2. The phenotype of *HB52* overexpression lines.

Figure S3. Root gravitropic response histogram of *HB52* knock-down mutants and overexpression lines.

Figure S4. Identification of *ctr1-1hb52* and *35S:EIN3-GFPPhb52*.

Table S1. Primers used in this study (5'- to -3').

Author Contributions

C.X. and Z.M. designed the experiments. Z.M., P.X., J.M., L.Y., Y.Y., and H.T. performed the experiments and data analyses. Z.M. wrote the manuscript. C.X supervised the project and revised the manuscript.

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References

- Abe, M., Katsumata, H., Komeda, Y., and Takahashi, T. (2003). Regulation of shoot epidermal cell differentiation by a pair of homeodomain proteins in Arabidopsis. *Development* **130**, 635-643.
- Alonso, J.M., and Ecker, J.R. (1999). EIN2, a Bifunctional Transducer of Ethylene and Stress Responses in Arabidopsis. *Science* **284**, 2148-2152.
- Alonso, J.M., Hirayama, T., Roman, G., Nourizadeh, S., and Ecker, J.R. (1999). EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. *Science* **284**, 2148-2152.
- Alonso, J.M., Stepanova, A.N., Solano, R., Wisman, E., Ferrari, S., Ausubel, F.M., and Ecker, J.R. (2003). Five components of the ethylene-response pathway identified in a screen for weak ethylene-insensitive mutants in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 2992-2997.
- An, F., Zhang, X., Zhu, Z., Ji, Y., He, W., Jiang, Z., Li, M., and Guo, H. (2012). Coordinated regulation of apical hook development by gibberellins and ethylene in etiolated Arabidopsis seedlings. *Cell research* **22**, 915-927.
- Aoyama, T., Dong, C.H., Wu, Y., Carabelli, M., Sessa, G., Ruberti, I., Morelli, G., and Chua, N.H. (1995).

394 Ectopic expression of the Arabidopsis transcriptional activator Athb-1 alters leaf cell fate in
395 tobacco. *The Plant cell* **7**, 1773-1785.

396 **Ariel, F.D., Manavella, P.A., Dezar, C.A., and Chan, R.L.** (2007). The true story of the HD-Zip family.
397 *Trends in plant science* **12**, 419-426.

398 **Benjamins, R., Quint, A., Weijers, D., Hooykaas, P., and Offringa, R.** (2001). The PINOID protein kinase
399 regulates organ development in Arabidopsis by enhancing polar auxin transport.
400 *Development* **128**, 4057-4067.

401 **Cai, X.T., Xu, P., Zhao, P.X., Liu, R., Yu, L.H., and Xiang, C.B.** (2014). Arabidopsis ERF109 mediates
402 cross-talk between jasmonic acid and auxin biosynthesis during lateral root formation. *Nat*
403 *Commun* **5**, 5833.

404 **Chang, K.N., Zhong, S., Weirauch, M.T., Hon, G., Pelizzola, M., Li, H., Huang, S.S., Schmitz, R.J., Urich,
405 M.A., Kuo, D., Nery, J.R., Qiao, H., Yang, A., Jamali, A., Chen, H., Ideker, T., Ren, B.,
406 Bar-Joseph, Z., Hughes, T.R., and Ecker, J.R.** (2013). Temporal transcriptional response to
407 ethylene gas drives growth hormone cross-regulation in Arabidopsis. *eLife* **2**, e00675.

408 **Chaves, A.L.S., and Mello-Farias, P.C.** (2006). Ethylene and fruit ripening: from illumination gas to the
409 control of gene expression, more than a century of discoveries. *Genetics & Molecular Biology*
410 **29**, 508-515.

411 **Delarue, M., Prinsen, E., Onckelen, H.V., Caboche, M., and Bellini, C.** (1998). Sur2 mutations of
412 Arabidopsis thaliana define a new locus involved in the control of auxin homeostasis. *The*
413 *Plant journal : for cell and molecular biology* **14**, 603-611.

414 **Dhonukshe, P., Huang, F., Galvan-Ampudia, C.S., Mahonen, A.P., Kleine-Vehn, J., Xu, J., Quint, A.,
415 Prasad, K., Friml, J., Scheres, B., and Offringa, R.** (2010). Plasma membrane-bound AGC3
416 kinases phosphorylate PIN auxin carriers at TPRXS(N/S) motifs to direct apical PIN recycling.
417 *Development* **137**, 3245-3255.

418 **Gao, Z., Chen, Y.F., Randlett, M.D., Zhao, X.C., Findell, J.L., Kieber, J.J., and Schaller, G.E.** (2003).
419 Localization of the Raf-like kinase CTR1 to the endoplasmic reticulum of Arabidopsis through
420 participation in ethylene receptor signaling complexes. *The Journal of biological chemistry*
421 **278**, 34725-34732.

422 **Grbić, V., and Bleeker, A.B.** (2003). Ethylene regulates the timing of leaf senescence in Arabidopsis.
423 *Plant Journal* **8**, 595-602.

424 **Ju, C., Yoon, G.M., Shemansky, J.M., Lin, D.Y., Ying, Z.I., Chang, J., Garrett, W.M., Kessenbrock, M.,
425 Groth, G., Tucker, M.L., Cooper, B., Kieber, J.J., and Chang, C.** (2012). CTR1 phosphorylates
426 the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to
427 the nucleus in Arabidopsis. *Proceedings of the National Academy of Sciences of the United*
428 *States of America* **109**, 19486-19491.

429 **Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A., and Ecker, J.R.** (1993). CTR1, a negative
430 regulator of the ethylene response pathway in Arabidopsis, encodes a member of the raf
431 family of protein kinases. *Cell* **72**, 427-441.

432 **Konishi, M., and Yanagisawa, S.** (2008). Ethylene signaling in Arabidopsis involves feedback regulation
433 via the elaborate control of EBF2 expression by EIN3. *The Plant journal : for cell and*
434 *molecular biology* **55**, 821-831.

435 **Le, J., Vandenbussche, F., Van Der Straeten, D., and Verbelen, J.P.** (2001). In the early response of
436 Arabidopsis roots to ethylene, cell elongation is up- and down-regulated and uncoupled from
437 differentiation. *Plant physiology* **125**, 519-522.

438 **Li, Z., Peng, J., Wen, X., and Guo, H.** (2013). Ethylene-insensitive3 is a senescence-associated gene
439 that accelerates age-dependent leaf senescence by directly repressing miR164 transcription
440 in Arabidopsis. *The Plant cell* **25**, 3311-3328.

441 **Mao, J.L., Miao, Z.Q., Wang, Z., Yu, L.H., Cai, X.T., and Xiang, C.B.** (2016). Arabidopsis ERF1 Mediates
442 Cross-Talk between Ethylene and Auxin Biosynthesis during Primary Root Elongation by
443 Regulating ASA1 Expression. *PLoS Genet* **12**, e1005760.

444 **Masucci, J.D., and Schiefelbein, J.W.** (1996). Hormones act downstream of TTG and GL2 to promote
445 root hair outgrowth during epidermis development in the Arabidopsis root. *The Plant cell* **8**,
446 1505-1517.

447 **Merchante, C., Alonso, J.M., and Stepanova, A.N.** (2013). Ethylene signaling: simple ligand, complex
448 regulation. *Current opinion in plant biology* **16**, 554-560.

449 **Nakamura, M., Katsumata, H., Abe, M., Yabe, N., Komeda, Y., Yamamoto, K.T., and Takahashi, T.**
450 (2006). Characterization of the class IV homeodomain-Leucine Zipper gene family in
451 Arabidopsis. *Plant physiology* **141**, 1363-1375.

452 **Petrasek, J., and Friml, J.** (2009). Auxin transport routes in plant development. *Development* **136**,
453 2675-2688.

454 **Pickett, F.B., Wilson, A.K., and Estelle, M.** (1990). The aux1 Mutation of Arabidopsis Confers Both
455 Auxin and Ethylene Resistance. *Plant physiology* **94**, 1462-1466.

456 **Prigge, M.J., and Clark, S.E.** (2006). Evolution of the class III HD-Zip gene family in land plants.
457 *Evolution & development* **8**, 350-361.

458 **Qiao, H., Shen, Z., Huang, S.S., Schmitz, R.J., Urich, M.A., Briggs, S.P., and Ecker, J.R.** (2012).
459 Processing and subcellular trafficking of ER-tethered EIN2 control response to ethylene gas.
460 *Science* **338**, 390-393.

461 **Ruzicka, K., Ljung, K., Vanneste, S., Podhorska, R., Beeckman, T., Friml, J., and Benkova, E.** (2007).
462 Ethylene regulates root growth through effects on auxin biosynthesis and
463 transport-dependent auxin distribution. *The Plant cell* **19**, 2197-2212.

464 **Sabatini, S., Beis, D., Wolkenfelt, H., Murfett, J., Guilfoyle, T., Malamy, J., Benfey, P., Leyser, O.,
465 Bechtold, N., Weisbeek, P., and Scheres, B.** (1999). An auxin-dependent distal organizer of
466 pattern and polarity in the Arabidopsis root. *Cell* **99**, 463-472.

467 **Sawa, S., Ohgishi, M., Goda, H., Higuchi, K., Shimada, Y., Yoshida, S., and Koshiba, T.** (2002). The
468 HAT2 gene, a member of the HD-Zip gene family, isolated as an auxin inducible gene by DNA
469 microarray screening, affects auxin response in Arabidopsis. *The Plant journal : for cell and*
470 *molecular biology* **32**, 1011-1022.

471 **Smalle, J., and Van Der Straeten, D.** (1997). Ethylene and vegetative development. *Physiologia*
472 *plantarum* **100**, 593-605.

473 **Stepanova, A.N., Hoyt, J.M., Hamilton, A.A., and Alonso, J.M.** (2005). A Link between ethylene and
474 auxin uncovered by the characterization of two root-specific ethylene-insensitive mutants in
475 Arabidopsis. *The Plant cell* **17**, 2230-2242.

476 **Stepanova, A.N., Robertson-Hoyt, J., Yun, J., Benavente, L.M., Xie, D.Y., Dolezal, K., Schlereth, A.,
477 Jurgens, G., and Alonso, J.M.** (2008). TAA1-mediated auxin biosynthesis is essential for
478 hormone crosstalk and plant development. *Cell* **133**, 177-191.

479 **Swarup, R., Perry, P., Hagenbeek, D., Van Der Straeten, D., Beemster, G.T., Sandberg, G., Bhalerao, R.,
480 Ljung, K., and Bennett, M.J.** (2007). Ethylene upregulates auxin biosynthesis in Arabidopsis
481 seedlings to enhance inhibition of root cell elongation. *The Plant cell* **19**, 2186-2196.

482 **Wen, X., Zhang, C., Ji, Y., Zhao, Q., He, W., An, F., Jiang, L., and Guo, H.** (2012). Activation of ethylene
483 signaling is mediated by nuclear translocation of the cleaved EIN2 carboxyl terminus. *Cell*
484 research **22**, 1613-1616.

485 **Won, C., Shen, X., Mashiguchi, K., Zheng, Z., Dai, X., Cheng, Y., Kasahara, H., Kamiya, Y., Chory, J.,**
486 **and Zhao, Y.** (2011). Conversion of tryptophan to indole-3-acetic acid by TRYPTOPHAN
487 AMINOTRANSFERASES OF ARABIDOPSIS and YUCCAs in Arabidopsis. *Proceedings of the*
488 *National Academy of Sciences of the United States of America* **108**, 18518-18523.

489 **Zhong, S., Zhao, M., Shi, T., Shi, H., An, F., Zhao, Q., and Guo, H.** (2009). EIN3/EIL1 cooperate with
490 PIF1 to prevent photo-oxidation and to promote greening of Arabidopsis seedlings.
491 *Proceedings of the National Academy of Sciences of the United States of America* **106**,
492 21431-21436.

493

494

495 **Figure legends**

496 **Figure 1. Expression pattern and subcellular localization of HB52.**

497 (A) Transcript level of *HB52* in different tissues. Seeds were germinated in the soil for
498 4 weeks then indicated tissues were collected to isolate RNA and detect the transcript
499 level of *HB52* by quantitative RT-PCR analysis. Values are mean $\pm$ SD (n=3
500 experiments).

501 (B-F) GUS staining of *HB52pro::GUS* transgenic plant. GUS activity was observed in
502 4-day old seedling (B), 10-day old seedling (E), 4-week adult seedling (F), root of
503 4-day old seedling (C, D). Plants were incubated in GUS staining solution for 2 hours
504 before photographs were taken. Bar=1cm in B, E, and F. Bar=100 μ m in C and D.

505 (G) Subcellular localization of the HB52 protein. *35S::HB52-GFP* transgenic seeds
506 were germinated on MS medium for 4 days then fluorescence was observed under
507 confocal laser scanning microscope.(Bar=100 μ m).

508

509 **Figure 2. *HB52* is responsive to ethylene and depends on ethylene signaling.**

510 (A) Transcript level of *HB52* in Col-0 and ethylene signaling mutants. Seeds were
511 germinated on MS medium for 4 days and then transferred to MS liquid medium
512 without or with 1 μ M ACC for 24 hours. Then RNA was isolated and quantitative
513 RT-PCR analysis was performed to detect the *HB52* expression level. Values are
514 mean $\pm$ SD (n=3 experiments, *P<0.05, **P<0.01, ***P<0.001). Statistically
515 significant differences were calculated based on the Student's *t*-tests.

516 (B-C) GUS staining of *HB52pro::GUS* transgenic seedlings in ethylene signaling
517 mutants. Seeds were germinated on MS medium for 4 days and then transferred to
518 MS liquid medium without or with 1 μ M ACC for 24 hours. Seedlings were incubated
519 in GUS staining solution for 0.5 hour before photographs were taken (B). The roots of
520 stained seedlings were observed under a microscope (C). Bar=1cm in B. Bar=100 μ m
521 in C.

522

523 **Figure 3. Primary root elongation of *HB52* knock-down mutants and**

524 **overexpression lines in response to ethylene.**

525 (A) *HB52* transcript levels in knock-down mutants and inducible overexpression lines.

526 Seeds were germinated on MS medium for 3 days and the seedlings were then
527 transferred to liquid MS medium with 5μM estradiol for 24 hours to induce gene
528 expression. Then roots were detached and RNA was isolated for quantitative RT-PCR
529 analysis subsequently. Values are mean ± SD (n=3 experiments, *P<0.05, **P<0.01,
530 ***P<0.001). Statistically significant differences were calculated based on the
531 Student's *t*-tests.

532 (B-C) Root elongation of knock-down mutants and inducible overexpression lines.

533 Seeds were germinated on MS medium for 3 days and then seedlings were transferred
534 to MS medium with 5μM estradiol to induce gene expression for 3 days. Afterwards,
535 seedlings were transferred to MS medium with 5μM estradiol supplemented with
536 0.1μM ACC, 1μM ACC, and 10μM ACC, respectively for 4 days. Then photographs
537 were taken (B) and primary root length were measured (C). Values are mean ± SD
538 (n=30 seedlings, *P<0.05, **P<0.01, ***P<0.001). Statistically significant
539 differences were calculated based on the Student's *t*-tests.

540

541 **Figure 4. Binding assays of EIN3, EIL1 proteins with the *HB52* promoter.**

542 (A) Schematic representation of *HB52* promoter showing putative EIN3 binding sites
543 (EBS) upstream of the transcription start site. EBS are indicated with yellow triangles
544 while black triangle indicates a control that has no EBS in this region. PCR-amplified
545 fragments are indicated by different pairs of colored primers used for ChIP-PCR and
546 quantitative ChIP-PCR.

547 (B-C) ChIP-PCR assays. 4-day old *35S:EIN3-GFP* and *35S:EIL1-GFP* transgenic
548 seedlings were treated with 1μM ACC for 24 hours for ChIP assays. About 200bp
549 *HB52* promoter fragments containing EBS were enriched by anti-GFP antibody in the
550 ChIP-PCR analysis (B). A region of *HB52* promoter which does not contain EBS was
551 used as a control. The results of ChIP-PCR were confirmed by quantitative
552 ChIP-PCR (C). Values are mean ± SD (n=3 experiments, *P<0.05, **P<0.01,
553 ***P<0.001). Statistically significant differences were calculated based on the

554 Student's *t*-tests.

555 (D) Yeast-one-hybrid assay. pGADT7/EIN3 (AD-EIN3) and pGADT7/EIL1

556 (AD-EIL1) constructs were co-transformed with pHIS2/HB52 (BD-cis) separately

557 into yeast strain Y187. AD/BD, AD/BD-cis1, AD/BD-cis2, AD/BD-cis3,

558 AD-EIN3/BD, AD-EIL1/BD were used as negative controls.

559

560 **Figure 5. *HB52* genetically acts downstream of *EIN3*.**

561 (A) Root elongation phenotype. Seeds of indicated lines were germinated on MS

562 medium without and with 1μM ACC for 5 days before photographs were taken.

563 Bar=1cm.

564 (B) Primary root length. Seeds of indicated lines as in (A) were germinated on MS

565 medium without and with 1μM ACC for 5 days before primary root length was

566 measured. Values are mean ± SD (n=30 seedlings, *P<0.05, **P<0.01, ***P<0.001).

567 Statistically significant differences were calculated based on the Student's *t*-tests.

568

569 **Figure 6. *HB52* affects auxin transport by regulating auxin transport-related** 570 **genes.**

571 (A) *HB52* transcript level of *HB52* mutants with DR5:GUS reporter. Seeds of

572 indicated lines were germinated on MS medium for 4 days and transferred to MS

573 liquid medium with 5 μM estradiol for 24 hours. Then roots were detached and RNA

574 was isolated for quantitative RT-PCR analysis subsequently. Values are mean ± SD

575 (n=3 experiments, *P<0.05, **P<0.01, ***P<0.001). Statistically significant

576 differences were calculated based on the Student's *t*-tests.

577 (B) GUS staining of DR5:GUS marker lines in varied *HB52* backgrounds. Seeds of

578 indicated lines were germinated on MS medium with 5 μM estradiol for 4 days and

579 transferred to liquid MS medium with 5 μM estradiol supplemented without and with

580 1μM ACC for 24 hours before staining. Seedlings were incubated in GUS staining

581 solution for 2 hours before photographs were taken. Bar=100μm

582 (C) Transcript level of auxin transport-related genes in mutants with varied *HB52*.

583 Seeds of indicated lines were germinated on MS medium for 4 days and transferred to

584 MS liquid medium with 5 μ M estradiol for 24 hours. Then roots were detached and
585 RNA was isolated for quantitative RT-PCR analysis subsequently. Values are mean $\pm$
586 SD (n=3 experiments, *P<0.05, **P<0.01, ***P<0.001). Statistically significant
587 differences were calculated based on the Student's t-tests.

588

589 **Figure 7. Binding assays of HB52 protein with the *WAG1* promoter.**

590 (A) Schematic representation of *WAG1* promoter with putative HB52 binding sites
591 upstream of the transcription start site. HB52 binding sites are indicated with yellow
592 and green triangles while black triangle indicates a control that has no HB52 binding
593 sites in this region. Numbers above the black lines represent the precise HB52 binding
594 sites. PCR-amplified fragments are indicated by different pairs of colored primers and
595 the primers are used to do quantitative RT-PCR.

596 (B) ChIP-PCR assay. 4-day old *35S:HB52-GFP* transgenic seedlings were treated
597 with 1 μ M ACC for the ChIP-PCR assay. A region of *WAG1* that does not contain
598 HB52 binding sites was used as a control. Values are mean $\pm$ SD (n=3 experiments,
599 *P<0.05, **P<0.01, ***P<0.001). Statistically significant differences were calculated
600 based on the Student's t-tests.

601 (C) Yeast-one-hybrid assay. pGADT7/HB52 (AD-HB52) was co-transformed with
602 pHIS2/*WAG1* (BD-w1) into yeast strain Y187. AD/BD, AD/BD-w1-1, AD/BD-w1-2,
603 AD/BD-w1-3, AD-HB52/BD were used as negative controls.

604 (D) EMSA of *in vitro* binding. Biotin-labelled probe (w1-1 region) was incubated
605 with HB52-MBP protein. As indicated, HB52-dependent mobility shifts were detected
606 and competed by the unlabeled probe in a dose-dependent manner.

607

608 **Figure 8. Binding assays of HB52 protein with the *WAG2* promoter.**

609 (A) Schematic representation of *WAG2* promoter with putative HB52 binding sites
610 upstream of the transcription start site. HB52 binding sites are indicated with yellow
611 triangles while black triangle indicates a control that has no HB52 binding sites in this
612 region. Numbers above the black lines represent the precise HB52 binding sites.
613 PCR-amplified fragments are indicated by different pairs of colored primers and the

614 primers are used to do quantitative RT-PCR.

615 (B) ChIP-PCR assay. 4-day old *35S:HB52-GFP* transgenic seedlings were treated

616 with 1 μ M ACC for the ChIP-PCR assay. A region of *WAG2* that does not contain

617 HB52 binding sites was used as a control. Values are mean $\pm$ SD (n=3 experiments,

618 *P<0.05, **P<0.01, ***P<0.001). Statistically significant differences were calculated

619 based on the Student's *t*-tests.

620 (C) Yeast-one-hybrid assay. pGADT7/HB52 (AD-HB52) was co-transformed with

621 pHIS2/*WAG2* (BD-w2) into yeast strain Y187. AD/BD, AD/BD-w2-1, AD/BD-w2-2,

622 AD-HB52/BD were used as negative controls.

623 (D) EMSA of *in vitro* binding. Biotin-labelled probe (w2-1 region) was incubated

624 with HB52-MBP protein. As indicated, HB52-dependent mobility shifts were detected

625 and competed by the unlabeled probe in a dose-dependent manner.

626

627 **Figure 9. Binding assays of HB52 protein with the *PIN2* promoter.**

628 (A) Schematic representation of *PIN2* promoter with putative HB52 binding sites

629 upstream of the transcription start site. HB52 binding sites are indicated with yellow

630 triangles while black triangle indicates a control that has no HB52 binding sites in this

631 region. Numbers above the black lines represent the precise HB52 binding sites.

632 PCR-amplified fragments are indicated by different pairs of colored primers and the

633 primers are used for quantitative ChIP-PCR.

634 (B) ChIP-PCR assay. 4-day old *35S:HB52-GFP* transgenic seedlings were treated

635 with 1 μ M ACC for the ChIP-PCR assay. A region of *PIN2* that does not contain HB52

636 binding sites was used as a control. Values are mean $\pm$ SD (n=3 experiments, *P<0.05,

637 **P<0.01, ***P<0.001). Statistically significant differences were calculated based on

638 the Student's *t*-tests.

639 (C) Yeast-one-hybrid assay. pGADT7/HB52 (AD-HB52) was co-transformed with

640 pHIS2/*PIN2* (BD-p2) into yeast strain Y187. AD/BD, AD/BD-p2-1, AD/BD-p2-2,

641 AD-HB52/BD were used as negative controls.

642 (D) EMSA of *in vitro* binding. Biotin-labelled probe (p2-1 region) was incubated with

643 HB52-MBP protein. As indicated, HB52-dependent mobility shifts were detected and

644 competed by the unlabeled probe in a dose-dependent manner.

645

646 **Figure 10. *PIN2*, *WAG1* and *WAG2* genetically act downstream of *HB52*.**

647 (A) *HB52* transcript level of varied *HB52* mutants. Seeds of indicated lines were
648 germinated on MS medium for 4 days, transferred to MS liquid medium with 5 μ M
649 estradiol and MS liquid medium with 5 μ M estradiol +1 μ M ACC for 48 hours. RNA
650 was isolated for quantitative RT-PCR analysis. Values are mean $\pm$ SD (n=3
651 experiments, *P<0.05, **P<0.01, ***P<0.001). Statistically significant differences
652 were calculated based on the Student's t-tests.

653 (B-C) Root elongation. Seeds of indicated lines were separately germinated on MS, 5
654 μ M estradiol and 5 μ M estradiol +1 μ M ACC for 5 days before photographs were
655 taken (B) (Bar=1cm). The primary root length was measured (C). Values are mean $\pm$
656 SD (n=30 seedlings, *P<0.05, **P<0.01, ***P<0.001). Statistically significant
657 differences were calculated based on the Student's t-tests.

658

659

A **B** **C** **D** **E** **F**

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G**Fluorescence****Bright****Merged**

A **B** **C**

A **B** **C** **D**

A

B

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C **D**

A

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C **B** **D**

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