PHB3 Regulates Lateral Root Primordia Formation via NO-mediated Degradation of AUX/IAAs

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Running title: PHB3 Regulates Lateral Root Primordia Development

Highlight: PHB3 regulates IAA28 and IAA14 degradation by controlling NO accumulation, and thereby regulating LR founder cell identification and the transition to asymmetric division.

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41 **Abstract**

42 We previously showed that *PHB3* regulates auxin-stimulated lateral root (LR)
 43 formation; however, the underlying molecular mechanism is unknown. Here, we
 44 demonstrate that *PHB3* regulates LR development mainly through influencing lateral
 45 root primordia (LRP) initiation via affecting nitric oxide (NO) accumulation. The
 46 reduced LRP in *phb3* was largely rescued by exogenous NO donor SNAP treatment.
 47 The decreased NO accumulation in *phb3* caused a lower expression of *GATA23* and
 48 *LBD16* through inhibiting the degradation of IAA14/28. Overexpression of either
 49 *GATA23* or *LBD16* in *phb3* mutant background recovered the reduced LRP number
 50 phenotype. These results indicate that PHB3 regulates LRP initiation via
 51 NO-mediated auxin signaling through regulating the degradation of IAA14/28.

52 **Key words:** PHB3, nitric oxide, auxin, lateral root primordia formation, *GATA23* and
 53 *LBD16*, molecular regulation

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55

56 **Introduction**

57 Root architecture is an important factor influencing crop yield. The plasticity of
58 lateral roots (LRs) allows the root architecture to adjust rapidly to the rhizosphere
59 environment, enhancing resistance to various stresses (De Smet *et al.*, 2012).
60 Signaling molecules, especially hormones and nitric oxide (NO), play important roles
61 in LR development (Fukaki & Tasaka, 2009; Jin *et al.*, 2011; Sun *et al.*, 2015).

62 Auxin signaling is critical for LR development and is mediated by the auxin
63 co-receptors TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX
64 (TIR1/AFB), the transcriptional repressors AUXIN/INDOLE-3-ACETIC ACID
65 (AUX/IAA), and AUXIN RESPONSE FACTORS (ARFs) (Correa-Aragunde,
66 Graziano, & Lamattina, 2004; Laskowski *et al.*, 2008; Fukaki & Tasaka, 2009;
67 Lavenus *et al.*, 2013; Guseman *et al.*, 2015). The auxin signaling cascade is activated
68 by the binding of auxin to the F-box auxin receptor complexes SCF^{TIR1/AFBs} followed
69 by ubiquitination of AUX/IAA proteins, which are then degraded by 26S proteasomes
70 (Lavy & Estelle, 2016; Leyser, 2018). LR initiation involves the specification and
71 asymmetric division of LR founder cells (De Rybel *et al.*, 2010; Goh, Joi, Mimura, &
72 Fukaki, 2012; Teixeira and Tusscher, 2019; Van Norman *et al.*, 2013). Of special
73 importance to LR initiation are IAA28 and IAA14. IAA28 plays a key role in
74 specifying pericycle cells for development into LR founder cells prior to LR initiation
75 (De Rybel *et al.*, 2010), while IAA14 is involved in the asymmetric division of LR
76 founder cells that occurs as part of LR initiation (Fukaki *et al.*, 2005; Goh, Joi,
77 Mimura, & Fukaki, 2012). IAA28 can interact with ARF5/6/7/8/19. When IAA28 is
78 degraded, ARF5/6/7/8/19 are free to activate *GATA23* expression, resulting in the
79 specification of LR founder cells (De Rybel *et al.*, 2010; Guseman *et al.*, 2015). Next,
80 auxin induces the degradation of IAA14/SLR, allowing ARF7/19 to activate the
81 expression of *LBD16*, which then promotes the asymmetric division required for the
82 migration of two adjacent founder cell nuclei to the common cell wall (Fukaki *et al.*,
83 2005; Goh, Joi, Mimura, & Fukaki, 2012). Then, the IAA14/SLR-ARF7/19 and
84 IAA12/BDL-ARF5 modules regulate the first round of asymmetric cell division and

85 initiate LR primordia (LRP) formation (Fukaki *et al.*, 2002; Okushima *et al.*, 2005;
86 Lavenus *et al.*, 2013).

87 The NO free radical has been reported to play important roles in a variety of
88 intracellular signaling in plants (Sengoku *et al.*, 2015). NO acts as a second
89 messenger that coordinates with hormone signaling to regulate growth, development,
90 and certain physiological processes (Zandonadi *et al.*, 2010; Terrile *et al.*, 2012; Arc
91 *et al.*, 2013). It has been found that auxin can promote NO accumulation and NO is
92 involved in auxin-induced LR development in tomato (*Solanum lycopersicum*) and
93 *Arabidopsis* (Correa-Aragunde, Graziano, & Lamattina, 2004; Kolbert *et al.*, 2008).
94 Research into the mechanisms by which NO regulates LR development has revealed
95 that NO influences auxin signaling through S-nitrosylation of ASK1 and TIR1 (Terrile
96 *et al.*, 2012; Iglesias *et al.*, 2018). Nitrosylation of ASK1 promotes the SCF^{TIR1/AFBs}
97 E3 ligase assembly (Iglesias *et al.*, 2018) and TIR1 S-nitrosylation can enhance the
98 interaction of TIR1/AFB with AUX/IAA, and thereby promote the degradation of
99 AUX/IAA proteins and the expression of auxin-induced genes (Terrile *et al.*, 2012).

100 Prohibitins (PHBs), originally discovered as tumor suppressors in mammalian
101 cells, are pleiotropic scaffold proteins involved in many important cell functions,
102 including survival, proliferation, metabolism, and mitochondrial dynamics (Wang *et*
103 *al.*, 2020) and exist widely in plants and animals. There are seven conserved *PHB*
104 genes in *Arabidopsis*, including *PHB3*, which is mainly localized in mitochondria and
105 involved in regulating plant development and stress responses (Van Aken *et al.*, 2007;
106 Wang *et al.*, 2010; Seguel *et al.*, 2018; Huang *et al.*, 2019). Dr. Larsen's group has
107 demonstrated that PHB3 plays an important role in modulating ethylene signaling in
108 *Arabidopsis* and that its mutant *eer3* shows reduced size of rosette leaves (Christians
109 & Larsen, 2007). Dr. Holuigue's group showed that PHB3 interacts with
110 ISOCHORISMATE SYNTHASE1 (ICS1), an essential enzyme in salicylic acid (SA)
111 biosynthesis, and that the *phb3* mutant exhibits reduced SA generation and impaired
112 responses to stresses such as pathogen infection (Seguel *et al.*, 2018).

113 In a previous study, we found that H₂O₂-induced NO accumulation was inhibited

114 in *phb3* mutants (Wang *et al.*, 2010). Furthermore, auxin-induced LR development
 115 was reduced in *phb3* mutants (Wang *et al.*, 2010). *PHB3* is mainly expressed in
 116 actively dividing tissues such as root tips, stems, and LRs (Kong *et al.*, 2018). When
 117 *ProAtPHB::GFP-GUS* transgenic plants, carrying a *GFP-GUS* reporter gene attached
 118 to the *PHB3* promoter, were treated with auxin 1-naphthaleneacetic acid (NAA),
 119 *PHB3* promoter activity could be detected in pericycle cells and was induced by NAA
 120 before the asymmetric division of founder cells (Van Aken *et al.*, 2007). These
 121 findings imply that *PHB3* may be involved in auxin-induced LR initiation and may be
 122 a key factor connecting endogenous NO accumulation and auxin-induced LR
 123 development, although the underlying mechanism is unknown.

124 Here, we demonstrate that *PHB3* regulates LRP initiation by controlling
 125 endogenous NO accumulation in LRPs. Treatments with NO induced the expression
 126 of *GATA23* and *LBD16*. However, *phb3* mutants showed reduced levels of *GATA23*
 127 and *LBD16* expression, and this reduction was correlated with strong inhibition of
 128 IAA28 and IAA14 degradation. These findings suggest that *PHB3* regulates
 129 NO-mediated degradation of IAA28 and IAA14 and affects LRP initiation by
 130 regulating the expression of *GATA23* and *LBD16*.

131

132 **Materials and Methods**

133 **Plant materials and *Arabidopsis* transformation**

134 The *Arabidopsis* mutant lines *gata23* (De Rybel *et al.*, 2010) and *lbd16*
135 (SALK_095791) and transgenic line *pLBD16::GUS* were provided by colleagues and
136 *phb3-3/ back26-2* mutant was generated as described by Wang *et al.* (2010). The
137 *phb3ko* (SALK_020707) mutant was ordered from the Arabidopsis Biological
138 Resource Center (ABRC) and verified by RT-PCR.

139 The *pPHB3::GUS* constructs were generated by introducing the histochemically
140 detectable β -glucuronidase (*GUS*) gene into the pPZP211 vector and inserting a
141 fragment of the *PHB3* promoter (2779 bp in front of ATG) upstream of *GUS*. The
142 *p35s::IAA14-GFP* and *p35s::IAA28-GFP* constructs were generated by inserting
143 fragments of the *IAA14* and *IAA28* open reading frames (ORFs) at the KpnI/SalI sites
144 upstream of a *GFP* ORF previously inserted into pPZP211. *p35s::GATA23* and
145 *p35s::LBD16* were generated by inserting the *GATA23* ORF and the *LBD16* ORF into
146 the pPZP211 vector. The primer sequences are given in Supplemental Table 1. These
147 constructs were transformed into *Agrobacterium tumefaciens* GV3101 and then into
148 WT or *phb3ko* mutant plants by the floral-dip method. The transgenic plants were
149 selected on 1/2 MS solid medium with 10 μ g/mL kanamycin.

150

151 **Phenotypic analysis**

152 *Arabidopsis* seeds were grown on 1/2 MS medium under long-day (LD; light: dark,
153 16 h: 8 h) conditions for 7 d, and then transferred into 1/2 MS medium added with 1
154 μ M IAA, 100 μ M SNAP, 500 μ M cPTIO, or 1 μ M NPA (or combinations thereof) for
155 another 2 d. The numbers of LR_s were then quantified and the length of PR_s per
156 seedling was measured under an Olympus BX53 fluorescence microscope. For
157 gravitation assay, the seeds of WT and *phb3* mutants were grown vertically on 1/2 MS
158 medium for 5 d and then rotated 90°. The LRP was observed under an Olympus BX53
159 fluorescence microscope 18 h later.

160

161 **GUS histochemistry**

162 Histochemical staining for *pGATA23::GUS*, *pLBD16::GUS* and *pPHB3::GUS*
163 activities in WT or *phb3* mutant plants was performed as previously described
164 (Sessions *et al.*, 1999) using 50 mM sodium phosphate buffer (pH 7.0) supplemented
165 with 0.2% (v/v) Triton- X-100, 10 mM potassium ferrocyanide, 10 mM potassium
166 ferricyanide, and 1 mM X-gluc. Samples were incubated overnight at 37°C and
167 examined for GUS staining under a dissecting microscope (Xu *et al.*, 2016).

168

169 **Endogenous NO detection in roots**

170 The seedlings grown on 1/2 MS medium were washed twice with deionized water and
171 incubated with 5 µM of the NO indicator DAF-FM DA for 15 min at room
172 temperature in the dark. Then the seedlings were washed with fresh buffer (Tris-HCl,
173 pH 7.0) for 15 min and endogenous NO was evaluated. DAF-FM DA reacts with NO
174 to produce strong fluorescence with an excitation wavelength of 495 nm and emission
175 wavelength of 515 nm. The endogenous NO was observed as green fluorescence
176 under an Olympus BX53 fluorescence microscope.

177

178 **Gene expression analysis**

179 Total RNA was extracted from the roots of seedlings previously treated with IAA or
180 SNAP using an Ultrapure RNA Kit (CWBIO, China) (Cao *et al.*, 2017). cDNA was
181 then synthesized using EasyScript One-Step gDNA Removal and cDNA Synthesis
182 SuperMix (TransGen, China). For qPCR, cDNA was conducted using a SYBR Green
183 Master qPCR Kit (Roche Diagnostics, USA). The relative expression levels were
184 calculated by the $\Delta\Delta C_T$ method (Schmittgen & Livak, 2008) using *ACT8* as the
185 reference gene.

186

187 **Transcriptome analysis**

188 *Arabidopsis* seeds were grown in 1/2 MS medium for 7 d. Total RNA of seedling
189 roots was extracted using an Ultrapure RNA Kit, and the concentrations were detected

190 using a NanoDrop 2000 spectrophotometer (Thermo). Three biological replicates
 191 were performed. The library was constructed and used for sequencing by Illumina
 192 HiSeq 2500 (GENE DENOVO, Guangzhou, China). For data processing, the clean
 193 reads were obtained from raw reads after filtering out adapters, poly(N), and
 194 low-quality reads. All the clean reads were then mapped to the *Arabidopsis* TAIR10.2
 195 reference genome using TopHat (version 2.0.12). The fragments per kilobase of exon
 196 per million fragments mapped (FPKM) value was used to evaluate gene transcripts. *P*
 197 value < 0.01, false discovery rate (FDR) < 0.01, and fold change ≥ 2 were used as the
 198 principle (Li *et al.*, 2017).

199

200 **Time-dependent fluorescence degradation assay**

201 For fluorescence degradation assay, 7-day-old seedlings grown in 1/2 MS liquid
 202 medium were treated with 25 μ M IAA and imaged with a Zeiss LSM880 (Zeiss)
 203 confocal laser-scanning microscope at 0, 5, 7, and 10 min after IAA treatment
 204 (Guseman *et al.*, 2015).

205

206 **Results**

207 **PHB3 is involved in LRP formation.**

208 To explore the molecular mechanism by which *PHB3* regulates LR development, two
 209 *phb3* mutants, *phb3-3* with a point mutation that converts Gly-37 to Asp and a T-DNA
 210 insertion line *phb3ko* (SALK_020707), were analyzed (Wang *et al.*, 2010). The plants
 211 grew on 1/2 MS medium for 7 d followed by measurement of LR. The results showed
 212 that the LR density was significantly lower in *phb3* mutants (Figure 1A and 1B). We
 213 further determined the LRP number and found that the density of LRP was also
 214 obviously decreased in *phb3* mutants than in WT (Figure 1C), indicating that *PHB3*
 215 plays an important role in LRP formation.

216 We also detected the expression of *PHB3* in WT roots using histochemical
 217 β -glucuronidase (GUS) analysis and found that *PHB3* was strongly expressed in the
 218 LRP (Figure 1D), consistent with its functions in LRP formation. To assess the LRP
 219 development of *phb3* mutants, we performed a gravitation assay, in which vertically
 220 cultured 5-day-old seedlings were rotated 90° and allowed to continue growing for 18
 221 h before phenotypic observation. Normally, it results in increased auxin concentration
 222 and a new LRP to be initiated on the outside of the root curve (Laskowski *et al.*, 2008;
 223 Guseman *et al.*, 2015). Our results showed that the LRP appeared clearly at the curve
 224 site of WT roots but the emergence of LRP was obviously inhibited in the *phb3*
 225 mutants (Figure 1E). Furthermore, we counted the number of the LRP stages of the
 226 plants (n=50) at 18 h after rotation growth. The statistical data showed that the LRPs
 227 in WT were mainly at the second, third and fourth stages of LRP development while
 228 those in the *phb3* mutants were predominantly inhibited or at the first stage (Figure
 229 1F). These results indicate that *PHB3* modulates the LRP formation.

230

231 **PHB3 regulates LRP initiation through NO.**

232 Our previous study has shown that both H₂O₂-induced NO accumulation and
 233 auxin-induced LR development are inhibited in *phb3* mutants (Wang *et al.*, 2010). To
 234 investigate the molecular mechanism of NO participating in auxin-induced LR

development, we analyzed the effects of NO on LR formation under our conditions. 5-day-old WT seedlings were treated with indole-3-acetic acid (IAA), auxin transport inhibitor naphthylphthalamic acid (NPA), NO donor S-nitroso-N-acetylpenicillamine (SNAP), and NO scavenger 2-(4-carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) for 2 d, and then assessed the LR density (Figure 2A and 2B). Exogenous IAA (1 μ M) or SNAP (100 μ M) treatments significantly increased the LR density of the seedlings, and application of both together had an even stronger effect, indicating that both IAA and NO promote LR formation under these conditions. Treatments with either NPA (1 μ M) or cPTIO (500 μ M) alone significantly reduced LR density and application of NPA and cPTIO together did not further enhance the reduction in LR density, suggesting that NO and IAA may function in the same pathway to regulate LR formation. Furthermore, treatment with SNAP restored LR formation in the NPA-treated seedlings while application of IAA did not rescue LR density in the cPTIO-treated seedlings, indicating that NO is required for auxin-induced LR development.

As NO regulate auxin-induced LR development, and H₂O₂-induced NO accumulation in *phb3* mutants is reduced, it is possible that *PHB3* affects NO accumulation and thereby modulates LR development. To test this hypothesis, we further detected auxin-induced endogenous NO accumulation in the roots of WT and *phb3* plants using the NO indicator diaminofluorescein-FM diacetate (DAF-FM DA), which produces strong fluorescence after reacting with NO (Namin *et al.*, 2013). The results showed that NO was mainly distributed around LRP, and the fluorescence of NO in the mutant was obviously weaker than that in WT (Figure 2C). After IAA treatment, the NO accumulation was strongly induced in the LRP of WT, while this induction was much lower in that of *phb3* mutants (Figure 2C). Additionally, application of NO donor SNAP increased LRP density of the *phb3* mutants (Figure 2D). These data suggest that *PHB3* participates in LRP development by mediating NO accumulation in plants.

To obtain molecular evidence of the effects of *PHB3* in regulating LR formation,

we performed RNA sequencing (RNA-seq) analysis of WT and *phb3* plants grown on 1/2 MS medium for 7 d. The results showed 4,294 differentially expressed genes (DEGs) with 2,065 up-regulated and 2,229 down-regulated ones in *phb3ko* (Figure 3A and File S1). GO enrichment analysis for these DEGs revealed clusters related to root development, auxin biosynthesis and response, and NO response (Figure 3B and File S2). Three root development-related clusters are root morphogenesis, LR development, and post-embryonic root development. Three auxin-related clusters were enriched: response to auxin, auxin biosynthetic process, and auxin metabolic process. Two NO-related clusters were also found: response to NO and cellular response to NO. These results provide further evidence that *PHB3* is involved in regulating NO- and auxin-induced LR formation.

***GATA23* and *LBD16* are involved in NO-induced LR development.**

To decipher further the mechanism by which NO regulates auxin-induced LR development, we investigated the transcript levels of four key genes involved in LRP formation (*GATA23*, *ARF19*, *LBD16*, and *LBD18*) in the roots of WT plants grown on 1/2 MS medium for 7 d. The results showed that the expression of *GATA23*, *ARF19*, *LBD16*, and *LBD18* was significantly induced by exogenous IAA (Figure 4A and Figure S1A), consistent with previous reports (Lavenus *et al.*, 2013). After SNAP treatment, the expression of *GATA23* and *LBD16* was found to be increased rapidly (Figure 4B), but no significant change was found for *ARF19* and *LBD18* (Figure S1B). Consistent with these results, GUS-staining assay revealed that the activity of the *GATA23* and *LBD16* promoter was greatly enhanced by SNAP treatment (Figure 4C). These data suggest that the expression of *GATA23* and *LBD16* is strongly induced by NO.

To test the effects of NO on the expression of genes in plants, we performed RNA-seq using extracts of the roots of WT plants treated with SNAP. The results showed that the expression of 3,194 (1653+1541) genes was altered after SNAP treatment (Figure 5A, File S3), among which many LR-regulated genes, including

293 IAA5, ILL6, PIN6, AIR12, IAA1, ARF16, PIN5, GATA23 and ILL3, showed
294 up-regulation (Figure 5B). GO analysis revealed that three auxin-related clusters were
295 enriched among these genes: response to auxin, auxin metabolic process, and auxin
296 biosynthetic process (Figure 5C, File S4). The above data suggest that NO may play
297 important roles in auxin signaling and metabolism.

298 We compared the DEGs between WT and *phb3* vs the SNAP-treated WT (Figure
299 5A), and found that 1541 DEGs in *phb3* mutant was regulated by NO, which is about
300 35.9% (1541/4294) of total DEGs in *phb3* mutant. Furthermore, 798 DEGs were
301 up-regulated by SNAP, while 94.6% (755/798) of 798 DEGs were down-regulated in
302 *phb3* mutant (Figure 5D). Meanwhile 743 were down-regulated by SNAP treatment,
303 among which 96.9% (720/743) were up-regulated in *phb3* mutant (Figure 5D). These
304 data indicate that most of genes up-regulated in *phb3* mutant are down-regulated in
305 SNAP-treated WT and vice versa. This opposite DEGs between SNAP treatment and
306 *phb3* mutant strongly suggest an important link between the function of PHB3 and
307 NO signaling.

308 Previous studies have shown that both *GATA23* and *LBD16* regulate LR
309 formation (De Rybel *et al.*, 2010; Goh *et al.*, 2012). Consistent with these findings,
310 we also found that LR development was inhibited in *gata23* and *lbd16* mutants
311 (Figure S2). To examine the roles of *GATA23* and *LBD16* in NO-induced LR
312 development, we investigated the effects of exogenous SNAP treatment on WT,
313 *gata23*, and *lbd16* plants. After a 2-d treatment with SNAP (100 μ M), the LR density
314 was strongly induced and increased 282% that of untreated control in the WT, but
315 only increased 43% in *gata23* and 149% in *lbd16*, respectively (Figure 6A and 6B).
316 These results indicate that both *GATA23* and *LBD16* contribute to NO-induced LR
317 formation.

318

319 **PHB3 regulates the expression of *GATA23* and *LBD16* by modulating** 320 **NO-mediated AUX/IAA degradation**

321 Considering that the accumulation of NO is strongly reduced in *phb3* mutants and that

the expression of LR regulatory genes (*GATA23* and *LBD16*) is enhanced by NO, we speculated that *PHB3* may influence LRP development by modulating the accumulation of NO. Therefore, we detected the expression of *GATA23* and *LBD16* by real-time qPCR. The results showed that the expression of *GATA23* and *LBD16* was significantly reduced in *phb3* plants (Figure 7A).

To determine whether *GATA23* and *LBD16* are functionally required for LRP initiation, we ectopically expressed *GATA23* and *LBD16* separately in the *phb3ko* mutant. The results showed increased the LRP density in transgenic plants of *GATA23* and *LBD16* genes (Figure 7B), indicating that both genes work downstream of *PHB3* to modulate LRP formation.

The auxin regulatory protein IAA28 plays a key role in specifying pericycle cells for development into LR founder cells prior to LR initiation (De Rybel *et al.*, 2010) and another protein, IAA14, is involved in the asymmetric division of LR founder cells that occurs as a part of LR initiation (Fukaki *et al.*, 2005; Goh *et al.*, 2012). Therefore, we investigated the transcript levels of *IAA28* and *IAA14* in *phb3* mutants. Interestingly, the expression of *IAA28* was significantly decreased and that of *IAA14* was increased in the mutants (Figure 7C), indicating that *PHB3* modulates the transcript levels of *IAA28* and *IAA14*. In theory, the decreased expression of *IAA28* should result in increased expression of *GATA23* (De Rybel *et al.*, 2010), which is the opposite of the reduced expression of *GATA23* found in *phb3* mutants. So, we further performed a protein degradation assay using fluorescent IAA-GFP protein, which has been widely used as an *in vivo* marker to detect the degradation of SCF^{TIR1}-AUX/IAA in the nucleus (Guseman *et al.*, 2016). In our assay, we transformed WT and *phb3* plants with the constructs *IAA28-GFP* and *IAA14-GFP*, respectively. The transgenic plants were grown on 1/2 MS solid medium for 7 d, and then treated with 25 μ M IAA. Fluorescence microscopy assessment showed that the *IAA28-GFP* and *IAA14-GFP* signals disappeared within 5 min of IAA treatment in the root cells of WT, but persisted in the root cells of *phb3* plants even after 10 min of IAA treatment (Figure 7D). In addition, we treated the *phb3* mutant with SNAP and found that the

351 degradation of IAA28 and IAA14 was restored after NO supply (Figure 7D). When
 352 treated with cPTIO, WT plants showed inhibited degradation of IAA28 and IAA14
 353 proteins (Figure 7D). These data indicate that NO plays a key role for the degradation
 354 of IAA28 and IAA14 and that PHB3 regulates LRP initiation by modulating
 355 NO-mediated AUX/IAA degradation.

356

357 Discussion

358 In nature, plants constantly adapt to the changing environment by adjusting their root
359 architecture. The LR is a critical factor determining root architecture (Nibau *et al.*,
360 2008). Understanding the regulatory mechanisms of LR development is thus of great
361 importance in efforts to enhance the yield and stress resistance of crops. Auxin plays
362 an essential role in LR development, including LRP initiation, LRP formation, and LR
363 growth (Fukaki *et al.*, 2005; De Rybel *et al.*, 2010; Lee *et al.*, 2013; Jeon *et al.*, 2017).
364 NO, as an important signaling molecule plays a crucial role in many plant
365 developmental processes including root development. NO has been found to regulate
366 auxin-induced LR development in tomato (Correa-Aragunde, Graziano, & Lamattina,
367 2004) and to promote the S-nitrosylation of TIR1, which enhances the interaction of
368 TIR1/AFB with AUX/IAA, resulting in the expression of IAA-induced genes (Terrile
369 *et al.*, 2012).

370 We previously showed that H₂O₂-induced NO accumulation is diminished and
371 the number of LRs is much lower in *phb3* mutants after auxin treatment (Wang *et al.*,
372 2010). However, the underlying mechanism whereby PHB3 regulates LR
373 development remained unclear. Here, we found that the density of LRPs was reduced
374 in *phb3* mutants, indicating that *PHB3* is required in LRP formation (Figure 1A-1C).
375 Furthermore, our data showed reduced number and the growth rate of LRPs in *phb3*
376 mutants (Figure 1A-F), indicating that *PHB3* regulates LR formation by influencing
377 the number and growth rate of LRPs.

378 Dr. Lamattina's group reported auxin-induced NO accumulation in tomato
379 pericycle cells undergoing anticlinal or periclinal division (Correa-Aragunde,
380 Graziano, & Lamattina, 2004), suggesting that NO plays an important role in the early
381 development of LRP. Application of the cPTIO inhibited auxin-induced LR formation.
382 When tomato plants were treated with NPA, NO treatment could still promote LR
383 initiation in the roots (Correa-Aragunde, Graziano, & Lamattina, 2004). We
384 investigated the effects of NO on auxin-induced LR formation using different
385 combination treatments of IAA, IAA transport inhibitor, NO donor, and NO scavenger

(Figure 2A-B). Our data indicate that NO plays an essential role in IAA-induced LR development, which is consistent with the previous results from tomato (Correa-Aragunde, Graziano, & Lamattina, 2004).

However, the mechanism by which PHB3 contributes to NO accumulation remains unknown. It has been demonstrated that PHB3 is dually localized in the mitochondria and chloroplasts of plant cells (Ahn *et al.*, 2006; Van Aken *et al.*, 2007; Seguel *et al.*, 2018). Both hydrogen peroxide (H_2O_2) and superoxide ion ($O_2^{\cdot-}$) have been found to accumulate in the mitochondria of *phb3* mutant cells (Kong *et al.*, 2018), indicating that PHB3 is involved in regulating the homeostasis of reactive oxygen species. Consistently, we performed RNA-sequencing on *phb3* mutant and GO analysis revealed two hydrogen peroxide-related clusters: regulation of hydrogen peroxide metabolic processes and hydrogen peroxide biosynthesis. In addition, NO has been shown to be consumed by reacting with $O_2^{\cdot-}$ in potato tuber mitochondria (de Oliveira *et al.*, 2008). In animals, excess $O_2^{\cdot-}$ can convert NO into ONOO⁻ and thereby decrease NO bioavailability (Brown & Borutaite, 2007; Schiffrin, 2008; Brandes *et al.*, 2011). We also found that the endogenous NO content was reduced in the roots of *phb3* mutants (Figure 2C). These findings implicate that the excess H_2O_2 and $O_2^{\cdot-}$ may convert NO to some intermediates, leading to the reduced accumulation of endogenous NO in *phb3* mutants.

In the efforts to decipher the mechanism of NO in regulating LR formation, we found that the expression of the root regulatory genes *GATA23* and *LBD16* can be induced by NO (Figure 4), suggest that NO regulates the LR development by promoting the specification of founder cells and the asymmetric division of founder cells. Further investigation revealed that the expression levels of these two genes were significantly decreased in the *phb3* mutants (Figure 7A), suggesting that *PHB3* may affect LRP formation by regulating the expression of these two genes. In addition, the LRP density of the *phb3* mutant could be rescued by over-expression of *GATA23* or *LBD16* (Figure 7B), indicating that *GATA23* and *LBD16* act downstream of *PHB3*.

414 These results suggest that PHB3 may regulate LR development by affecting the
415 accumulation of NO and then modulating the expression *GATA23* and *LBD16*.

416 As the expression of *GATA23* and *LBD16* is regulated by IAA28 and IAA14,
417 respectively (Lavenus *et al.*, 2013), we investigated IAA28 and IAA14 expression in
418 the *phb3* mutants. The expression of IAA28 was inhibited while the expression of
419 IAA14 was increased in *phb3* mutants (Figure 7C). Decreased expression of IAA28
420 should result in increased expression of *GATA23*. But actually, reduced expression of
421 *GATA23* was found in *phb3* mutants. These results implicate that the regulation of
422 *GATA23* by IAA28 may not at the transcriptional levels but at protein levels. In
423 addition, NO can promote the degradation of AUX/IAA proteins through enhancing
424 the interaction between TIR1/AFB and AUX/IAA (Terrile *et al.*, 2012). However, the
425 mechanism by which AUX/IAA proteins participate in NO-regulated LR development
426 was still unclear. Therefore, we performed AUX/IAA protein degradation assays and
427 found that IAA28-GFP and IAA14-GFP degradation was strongly delayed in *phb3*
428 mutant after IAA treatment (Figure 7D). Furthermore, the degradation of both
429 proteins was restored in *phb3* mutant after NO treatment. Thus, the decreased
430 expression of *GATA23* is caused by NO-mediated delay of IAA28 protein degradation
431 and not by decreased gene expression of IAA28. Similarly, the decreased gene
432 expression of *LBD16* results from delayed degradation of IAA14 protein.

433 Taken together, our findings suggest that *PHB3* regulates the degradation of
434 IAA28 and IAA14 by controlling endogenous NO concentrations, thereby regulating
435 the expression of downstream genes (*GATA23* and *LBD16*) and ultimately modulating
436 the founder cell identity and asymmetric division (Figure 8). The *PHB3*-mediated
437 regulation of AUX/IAA proteins and the downstream genes *GATA23* and *LBD16*
438 provides insight into the cross-talk between NO and auxin-induced LR formation and
439 a new mechanism of IAA-mediated LR development.

440

441 **Supplementary data**

442 **Supplemental Table S1. Primer list**

443 **Supplemental Figure S1.** The expression of the LR regulatory genes *ARF19* and
444 *LBD18* after IAA and SNAP treatment.

445 **Supplemental Figure S2.** LR development is inhibited in *gata23* and *lbd16* mutants.

446 **Supplemental File S1.** Genes whose expression changed more than 2-fold in WT and
447 *phb3* mutant.

448 **Supplemental File S2.** Some clusters for genes differentially expressed in WT and
449 *phb3* mutant.

450 **Supplemental File S3.** Genes whose expression changed more than 2-fold after NO
451 treatment.

452 **Supplemental File S4.** Some clusters for genes differentially expressed in WT and
453 SNAP-treated.

454

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459

460 **Author Contributions**

461 Y.W. designed the research. S.L., Q.L., X.T., L.M., M.L., X.W., N.L., F.L., and J.S.
462 performed the experiments. Q.L., Y.W., and S.L. analyzed the data. Y.W., N.M.C,
463 and Q.L. wrote the manuscript.

464

465 **Disclosures**

466 The authors have no conflicts of interest to declare

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Figures

Figure 1. PHB3 is involved in LRP initiation.

(A) LR growth in WT and *phb3* plants. Plants were grown on 1/2 MS medium for 7 d. Scale bars, 5 mm.

(B) LR density in WT and *phb3* mutants. Plants were grown under the same conditions as in (A). LR density is the number of emerged LRs per 1 cm of the primary root length. Student's *t*-test, ** $P < 0.01$, * $P < 0.05$. The data are presented as means $\pm$ SD ($n \geq 20$).

(C) LRP density in WT and *phb3* plants. Plants were grown on 1/2 MS medium for 7 d. LRP density is the number of emerged LRs per 1 cm of the primary root length. Student's *t*-test, ** $P < 0.01$. The data are presented as means $\pm$ SD ($n \geq 20$).

(D) The promoter activity of *PHB3* in the LRP different stages. Plants were grown on 1/2 MS solid medium for 7 d and GUS staining was detected. Scale bars, 50 μ m.

(E) LRP initiation in WT and *phb3* mutants. Plants were grown vertically on 1/2 MS medium for 7 d and then rotated 90°. LRP were counted using an Olympus BX53 fluorescence microscope after 18 h of rotation. The arrows represent LRP. Scale bars, 50 μ m.

(F) The quantitative results of (E), $n = 50$.

Figure 2. PHB3 regulates LRP initiation through NO.

(A) WT plants were grown hydroponically in 1/2 MS medium for 5 d and then treated with various combinations of SNAP (100 μ M), IAA (1 μ M), cPTIO (500 μ M), and NPA (1 μ M) for 2 d. Scale bar, 5mm.

(B) Quantitative data for LR density in WT plants after SNAP (100 μ M), IAA (1 μ M), cPTIO (500 μ M), and NPA (1 μ M) treatments. Student's *t*-test, ns, not significant difference, ** $P < 0.01$, and *** $P < 0.001$. The data are presented as means $\pm$ SD ($n \geq 20$).

(C) NO accumulation in WT and *phb3* mutants. Plants were grown on 1/2 MS medium for 7 d. The seedlings were then washed twice with pure water and incubated with 5 μ M DAF-FM DA for 15 min at room temperature in the dark. NO appeared as green fluorescence and was observed in primary roots (PR) and LRPs using an Olympus BX53 fluorescence microscope. Scale bars, 50 μ m.

(D) LRP density in NO-treated WT and *phb3ko* seedlings. Plants were grown on 1/2 MS medium for 5 d and then treated with 100 μ M SNAP for 2 d. Student's *t*-test, ** $P < 0.01$, *** $P < 0.001$. The data are presented as means $\pm$ SD ($n \geq 20$).

Figure 3. RNA-seq analysis for genes differentially expressed in WT and *phb3* plants.

(A) Genes differentially expressed in WT and *phb3*. Plants were grown on 1/2 MS medium for 7 d, and then the roots were collected for RNA extraction used in RNA-sequencing.

(B) GO analysis for genes differentially expressed in WT and *phb3* plants.

Figure 4. *GATA23* and *LBD16* are transcriptionally regulated by NO.

(A) WT plants were grown on 1/2 MS medium for 7 d, and then treated with 1 μM IAA for 0 h, 0.5 h and 1 h, respectively, after which the roots were collected for RNA extraction. The expression of the *GATA23* and *LBD16* genes was determined by qPCR. Student's *t*-test, *** $P < 0.001$. The data are presented as means $\pm$ SD ($n = 5$).

(B) WT plants were grown on 1/2 MS medium for 7 d and then treated with 100 μM SNAP for 0 h, 0.5 h and 1 h, respectively, after which the roots were collected for RNA extraction. The expression of *GATA23* and *LBD16* was determined by qPCR. Student's *t*-test, * $P < 0.05$, *** $P < 0.001$. The data are presented as means $\pm$ SD ($n = 5$).

(C) Promoter activities of *GATA23* and *LBD16* in WT. Plants were grown on 1/2 MS medium for 7 d and then treated with 50 μM SNAP for 2 h. The promoter activities of the two genes are indicated by arrows. Scale bars, 50 μm.

Figure 5. Analysis for DEGs in WT after NO treatments and DEGs in *phb3* mutant.

(A) Venn diagram showing the number of genes differentially expressed in *phb3* mutant, and in WT roots by treatments with different concentrations of SNAP (50 μ M) for 1 h compared with control plants (0 μ M SNAP). Plants were grown on 1/2 MS medium for 7 d, and then treated with different concentrations of SNAP (0 μ M and 50 μ M) for 1 h, after which the roots were collected for RNA extraction used in RNA-sequencing.

(B) Known LR-regulated genes with DEGs in in WT after SNAP treatment.

(C) GO analysis for DEGs in WT after SNAP treatment.

(D) Transcriptome analysis of WT and *phb3* vs the SNAP-treated WT.

Figure 6. *GATA23* and *LBD16* are involved in NO-induced LR development.

(A) WT, *gata23*, and *lbd16* plants were grown on 1/2 MS liquid medium for 5 d, and then treated with SNAP (100 μ M) for 2 d. Scale bars, 5 mm.

(B) Density of LRs was quantified by microscopy observation. Student's *t*-test, *** $P < 0.001$. The data are presented as means $\pm$ SD ($n \geq 20$).

Figure 7. *PHB3* affects the expression of *GATA23* and *LBD16* through NO-mediated degradation of *IAA14/28*.

(A) Expression of *GATA23* and *LBD16* in WT and *phb3* mutants. Plants were grown on 1/2 MS medium for 7 d and then roots were collected for RNA extraction. The expression of the genes was determined by qPCR. Student's *t*-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. The data are presented as means $\pm$ SD ($n = 5$).

(B) LRP density in WT, *GATA23/phb3ko*, and *LBD16/phb3ko* plants. Plants were grown on 1/2 MS medium for 7 d. Student's *t*-test, *** $P < 0.001$. Data are presented as means $\pm$ SD ($n = 20$).

(C) Expression of *IAA28* and *IAA14* in WT and *phb3* mutants. Plants were grown on 1/2 MS medium for 7 d and then roots were collected for RNA extraction. The expression of the two genes was determined by qPCR. Student's *t*-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. The data are presented as means $\pm$ SD ($n = 5$).

(D) Degradation of IAA28-GFP and IAA14-GFP in the roots of WT and *phb3ko* mutant. Plants were grown on 1/2 MS medium for 7 d and then treated with 25 μ M IAA, 25 μ M IAA +500 μ M cPTIO, and 25 μ M IAA +100 μ M SNAP, respectively. The green fluorescence of IAA-GFP was observed with a Zeiss LSCM high-resolution laser confocal microscope. Scale bars, 500 μ m.

Figure 8. Model showing the roles of *PHB3* in regulating LRP initiation.

PHB3 regulates LRP initiation by controlling the levels of NO. NO may promote the IAA-mediated degradation of IAA28 and release of ARFs. Released ARFs can bind to the promoter and activate the expression of downstream gene *GATA23* to control LR founder cell specification. Then NO may promote the degradation of IAA14 and release of its interacted ARFs. The released ARFs can bind to the promoter and activate the expression of the *LBD16* to initiate asymmetric division of LR founder cells.