BIG Modulates Stem Cell Niche and Meristem Development via SCR/ShR Pathway in Arabidopsis Roots

Zhongming Liu 1,2, Ruoxi Zhang 1, Wen Duan 1,2, Baoping Xue 1,2, Xinyue Pan 1, Shuangchen Li 1, Peng Sun 1, Limin Pi 3,* and Yun-Kuan Liang 1,2,*

1 State Key Laboratory of Hybrid Rice, Department of Plant Sciences, College of Life Sciences, Wuhan University, Wuhan 430072, China; zhangruoxi@wbgcas.cn (R.-X.Z.); duanwen@whu.edu.cn (W.D.); xuebaoping@whu.edu.cn (B.X.); 2018202040084@whu.edu.cn (X.P.); lishuangchen2012@163.com (S.L.); 00033070@whu.edu.cn (P.S.)
2 Hubei Hongshan Laboratory, Wuhan 430007, China
3 State Key Laboratory of Hybrid Rice, The Institute for Advanced Studies, Wuhan University, Wuhan 430072, China
* Correspondence: limin.pi@whu.edu.cn (L.P.); ykliang@whu.edu.cn (Y.-K.L.)

Abstract: BIG, a regulator of polar auxin transport, is necessary to regulate the growth and development of Arabidopsis. Although mutations in the BIG gene cause severe root developmental defects, the exact mechanism remains unclear. Here, we report that disruption of the BIG gene resulted in decreased quiescent center (QC) activity and columella cell numbers, which was accompanied by the downregulation of WUSCHEL-RELATED HOMEOBOX5 (WOX5) gene expression. BIG affected auxin distribution by regulating the expression of PIN-FORMED proteins (PINs), but the root morphological defects of big mutants could not be rescued solely by increasing auxin transport. Although the loss of BIG gene function resulted in decreased expression of the PLT1 and PLT2 genes, genetic interaction assays indicate that this is not the main reason for the root morphological defects of big mutants. Furthermore, genetic interaction assays suggest that BIG affects the stem cell niche (SCN) activity through the SCR/SCARECROW (SCR)/SHORT ROOT (SHR) pathway and BIG disruption reduces the expression of SCR and SHR genes. In conclusion, our findings reveal that the BIG gene maintains root meristem activity and SCN integrity mainly through the SCR/SHR pathway.

Keywords: BIG; stem cell niche; polar auxin transport; SHORT ROOT; SCARECROW; PLETHORA

1. Introduction

In Arabidopsis (Arabidopsis thaliana L.), the indeterminate growth of the root is supported by a series of undifferentiated stem cells, which are located in the root apical meristem (RAM). The RAM pattern is formed by a range of asymmetric divisions of stem cells around the quiescent center (QC) that is almost undivided under normal conditions [1–3]. These stem cells, together with the QC in contact with them, make up the stem cell niche (SCN), which supplies the source of cells for RAM [1–3]. Post-embryonically, the expression of the WUSCHEL-RELATED HOMEOBOX5 (WOX5) gene is strictly limited to QC for defining QC and maintaining the undifferentiated condition of columella stem cells (CSCs) [4,5]. Loss of WOX5 gene function results in the differentiation of CSCs that displays the accumulation of starch, which is also accompanied by an additional division of the QC [4,5].

The SCR/SCARECROW (SCR)/SHORT ROOT (SHR) and PLETHORA (PLT) pathways are two independent pathways involved in the regulation of SCN activity and root meristem size [6]. Both SHR and SCR are members of the GRAS transcription factor family that are necessary for the maintenance of SCN activity [6,7]. SHR is synthesized in the stele and then transported to the neighboring cell layer [8], which nucleates the assembly of the SHR/SCR heterocomplex [9–13]; this heterocomplex promotes SHR nuclear localization and increases...
SCR expression, which in turn constrains the movement of the SHR protein to the next layer of cells [8, 11]. Both shr and scr mutants display defective QC maintenance and fail to maintain root meristem activity that leads to a phenotype of shorter meristems [8, 11, 14]. Mutations in the SCR and SHR genes cause the cortex/endodermis initially to fail to complete normal asymmetric division, resulting in the formation of only one layer of ground tissue [9–13].

Auxin and its gradient distribution play important roles in a wide range of growth and development processes, including embryogenesis, organogenesis, cell determination and division, tissue patterning, and tropisms in plants [15–18]. Additionally, auxin controls proper root pattern formation and SCN maintenance [16, 19]. The directional transport of auxin allows for the asymmetric distribution of auxin in distinct cells and tissues [15] to generate local auxin maxima, minima, and gradients for organ initiation and shape determination [16, 17]. The auxin-inducible PLETHORA (PLT1 and PLT2) genes, which encode two members of the AP2 class transcription factors, are required for the transcription of numerous root development-related genes, including genes that are involved in auxin biosynthesis and transport [20–22]. plt1 plt2 double mutants cannot resolve correct QC morphology and show a dramatic reduction in root meristem length [20, 23]. Interestingly, loss of PLT1 and PLT2 function did not affect SCR and SHR expression, although plt1 plt2 mutants, scr, and shr mutants all exhibited SCN defects [20], suggesting that the PLTs pathway and the SHR/SCR pathway are parallel and converge to maintain the SCN [20–22].

Ubiquitin protein ligase E3 component N-recognin 4 (UBR4) is a 600-kDa calmodulin-interacting protein involved in the N-end rule pathway [24–27]. BIG was originally identified in a mutational screen for resistance against N-1-naphthylphthalamic acid (NPA), a potent auxin transport inhibitor [28]. BIG is the only UBR4 homolog in Arabidopsis [29], which has been demonstrated to modulate circadian adjustment [30] and C/N balance [31]. Mutations in the BIG gene result in multiple defects, including altered aerial organ development, impaired hormone and light signaling, root growth and lateral root development, and aberrant stomatal CO₂ responses and immunity [28, 32–38].

Previous work has shown that the BIG gene is involved in regulating polar auxin transport and big mutants show severe root developmental defects [28, 29, 35]. In this study, we sought to develop a better understanding of how the BIG gene is involved in the root development and found that the BIG gene is integral to the maintenance of SCN and meristem activity, and this function depends on the SCR/SHR pathway. Mutations in the BIG gene lead to a severe reduction in meristem activity and reduced QC activity. Genetic analysis shows that the WOX5 gene has epistatic effects on the BIG gene in maintaining SCN. Decreased levels of PIN-FORMED proteins (PINs) are likely a part of the reasons for the auxin transport defects in big mutants, though increasing auxin transport could not rescue phenotypic defects in big mutant roots. Loss of BIG function results in the downregulation of PLTs gene expression, whereas the big mutant has additive effects with the plt1-4 plt2-2 mutants on the control of root growth. Further genetic experiments show that BIG regulates root development in an SCR/SHR-dependent manner. Consistently, the expression of SCR and SHR genes was reduced in the big mutants. Taken together, we propose that the BIG gene participates in maintaining SCN and meristem activity through the SCR/SHR pathway.

2. Results

2.1. The BIG Gene Is Involved in the Maintenance of SCN Integrity

As evident from previous work, big mutants showed a much-reduced root length compared to the wild-type (WT, Col-0) seedlings at 5 days after germination (DAG) [28–35]. We questioned whether BIG participates in the maintenance of SCN. The expression of QC46 [5], which is a QC-specific marker, was used to measure QC activity in WT and big-1. The slightly decreased expression of QC46 in big-1 indicates that BIG participates in maintaining the activity of the QC (Figure 1A). Endoplasmic reticulum (ER)-localized GFP driven by the WOX5 promoter (pWOX5:erGFP) [39] were crossed into big mutants
before confocal laser scanning microscopy (CLSM) was performed, and the results show that mutations in the BIG gene caused a reduction in WOX5 transcription (Figure 1B,C). In line with this observation, mRNA quantification by the reverse transcription-quantitative PCR (RT-qPCR) revealed a significant downregulation of the WOX5 expression in big-1 (Figure 1D).

**Figure 1.** BIG is involved in maintaining the SCN. (A) QC46 expression in WT and big-1 seedlings at 5 DAG. Scale bars, 20 µm. (B) pWOX5:erGFP expression in WT and big seedlings at 5 DAG. Left, the merge of the PI and GFP channels; right, the GFP channel displayed in pseudo colors with intensity scale. a.u. indicates arbitrary units. Scale bars, 20 µm. (C) Quantification of pWOX5:erGFP relative intensity in WT and big seedlings at 5 DAG. Values are means ± SD (n ≥ 30). One-way ANOVA, Tukey’s multiple comparisons test. Different letters indicate values are significantly different (p < 0.01). (D) qRT-PCR analyses show the relative expression levels of WOX5 in WT and big seedlings. Error bars represent SD. Student’s t-test. Different letters indicate values are significantly different (p < 0.01). (E) mPS-PI staining of WT, big-1, wox5-1, and wox5-1 big-1 root tips at 5 DAG. Scale bars, 50 µm. (F) Quantification of columella cell layer number in WT and big seedlings at 5 DAG. Values are means ± SD (n ≥ 30). One-way ANOVA, Tukey’s multiple comparisons test. Different letters indicate values are significantly different (p < 0.01).

Given that WOX5 is indispensable for maintaining the CSCs in an undifferentiated state [4,5], we next wanted to know whether the reduction of WOX5 expression in big mutants affects the development of CSCs and the derived columella cells. As shown in Figure 1E,F, the columella cells in big mutants had one layer less than those of WT, suggesting that BIG deficiency disturbed the development of CSCs and columella cells. In addition, to verify the genetic relationship between the WOX5 gene and the BIG gene, wox5-1 [4] was introduced into big-1, and the phenotypes of the SCN were monitored. As shown in Figure 1E, the modified pseudo-Schiff propidium iodide (mPS-PI) staining of the wox5-1 big-1 double mutant showed a similar QC phenotypic defect to wox5-1 but more severe than that of big-1, indicating that the WOX5 gene has epistatic effects on the BIG gene.
in maintaining SCN. These results indicate that the BIG gene is involved in maintaining SCN integrity.

2.2. Increasing the Auxin Transport Capacity Cannot Rescue the Phenotypic Defects of Big Mutants

The BIG gene is required for regulating the polar auxin transport and the endocytosis, two processes that involve the PIN protein family [28,29,32,34]. To determine the auxin accumulation of big mutant in root tips, seedlings expressing GUS under the control of the artificial DR5 promoter were subjected to examine the concentrations of auxin. As shown in Figure 2A, the GUS staining signals of pDR5::GUS [40] in the root tip of big-1 were significantly less than those of WT, suggesting that fully functional BIG is required for the proper auxin accumulation, in agreement with several previous studies [28,29,32,34]. Given that BIG acts synergistically with PIN1 to control the development of leaves and shoot apical meristems [35], we hypothesized that BIG regulates polar auxin transport by regulating the expression of the PIN proteins. To this end, big-1 was introduced into the transgenic plants that express translational fusions of PIN:GFP proteins under the control of the native promoter of each individual PIN gene. The fluorescence intensity of pPIN1::PIN1:GFP [41], pPIN2::PIN2:GFP [16], pPIN3::PIN3:GFP [16], and pPIN7::PIN7:GFP [16] was significantly reduced by a big-1 mutant (Figure 2C–J), suggesting that BIG influences the polar auxin transport via modulating PINs expression. To further ascertain this observation, RT-qPCR analysis was conducted, which showed great reductions in PINs transcription in big-1 compared to that of WT (Figure 2B). Together, these findings indicate the capacity of BIG for regulating the expression of PINs family proteins and influencing the polar auxin transport.

Strigolactones modulate not only shoot branching but also the root architecture, such as lateral root formation, root hair elongation, and meristem cell number through a variety of MORE AXILLARY GROWTH (MAX) genes that positively regulate auxin transport [42–45]. The max4-1 mutant was used to efficiently rescue the auxin transport deficiency of tir3-101 (another allele mutant of the BIG gene) but failed to alleviate the over-branched phenotype of tir3-101 [42]. We wanted to know whether the suppressive effect on auxin transport by max4-1 could restore the RAM defects in big mutants. As shown in Figure S1B,C, the root morphological structure of the max4-1 tir3-101 double mutant [42] displayed no detectable difference from that of tir3-101. Then, the max4-1 big-1 double mutant expressing pDR5rev::GFP [46] was generated to assess auxin concentrations in root tips. As shown in Figure 3A,B, the fluorescence intensity of pDR5rev::GFP in max4-1 was higher than that of WT, but there was no significant difference in the fluorescence intensity between the max4-1 big-1 double mutant and the WT, suggesting that max4-1 increased the auxin transport capacity in root tips in the big-1 mutant. Intriguingly, as shown in Figure 3C–F, compared to big-1, the root morphology was not restored in the max4-1 big-1 double mutant, suggesting that the morphological defects of big mutants could not be rescued by manipulating the auxin transport. To further evaluate whether increasing auxin content in big mutants could alter the root morphology, big-1 was crossed with the YUCCA-OX transgenic plants that overexpress YUCCA1, a key auxin biosynthesis gene [47]. YUCCA-OX big-1 seedlings had longer hypocotyl and petioles relative to big-1. In contrast, YUCCA-OX big-1 showed no discernable restoration of root morphology compared to big-1 (Figure S1D–F), suggesting that improving the capacity of auxin transport could not affect the RAM phenotypes in big mutants.

2.3. BIG and PLT Genes Function Independently in Regulating Root Growth

Auxin-induced gradients of PLTs (PLT1–4) converge at the SCN to form a concentration peak and maintain the stem cell identity [20–23]. Since restoring the impaired auxin transport capacity could not affect the root phenotypes in the big mutant, we hypothesized that BIG deficiency might disturb the auxin-related PLT pathway. The plt1-4 plt2-2 [20] double mutant was crossed with the big-1 mutant. Data in Figure 4A–D show that the length of either root or of the RAM in the plt1-4 plt2-2 big-1 triple mutant was significantly shorter
than that in the plt1-4 plt2-2 double mutant. Moreover, the meristematic cells of the plt1-4 plt2-2 big-1 triple mutants appeared to be completely differentiated, in marked contrast to the plt1-4 plt2-2 double mutants (Figure 4C), indicating that big mutant has additive effects with plt1-4 plt2-2 on the control of root growth. Next, we crossed the big-1 mutant with the transgenic plants that express ER-localized cyan fluorescent protein (CFP) under the control of the PLT promoters. The fluorescence intensities of pPLT1::erCFP [23] and pPLT2::erCFP [23] were lower in big-1 compared to WT seedlings (Figure 4E–H), indicating that mutation in the BIG gene decreased the expression of the PLT genes. RT-qPCR analysis of the PLT genes lent further support to this outcome (Figure 4I). From this evidence, we conclude that the PLT pathway is not the main cause of the root morphological defects in the big mutant.

**Figure 2.** BIG is involved in the regulation of polar auxin transport. (A) pDR5::GUS expression in WT and big seedlings at 5 DAG. Scale bar, 20 µm. (B) qRT-PCR analyses show the relative expression levels of PIN1, PIN2, PIN3, and PIN7 in WT and big-1 seedlings. Error bars represent SD. Two-way ANOVA, Tukey’s multiple comparisons test. Different letters indicate values are significantly different ($p < 0.05$). (C,E,G) pPIN1::PIN1::GFP (C), pPIN2::PIN2::GFP (E), pPIN3::PIN3::GFP (G), and pPIN7::PIN7::GFP (I) expression in WT and big-1 seedlings at 5 DAG. Scale bar, 50 µm. (D,F,H,J) Quantification of pPIN1::PIN1::GFP (D), pPIN2::PIN2::GFP (F), pPIN3::PIN3::GFP (H), and pPIN7::PIN7::GFP (J) relative intensity in WT and big-1 seedlings at 5 DAG. Values are means ± SD ($n \geq 30$). Student’s $t$-test. Different letters indicate values are significantly different ($p < 0.01$).
Figure 3. Increasing auxin could not restore the root morphology in big mutants. (A) pDR5rev::GFP expression in WT and big seedlings at 5 DAG. Scale bar, 16 µm. (B) Quantification of pDR5rev::GFP relative intensity in WT, big-1, max4-1, and max4-1 big-1 seedlings at 5 DAG. Values are means ± SD (n ≥ 25). One-way ANOVA, Tukey’s multiple comparisons test. Different letters indicate values are significantly different (p < 0.01). (C) Images of the indicated genotypes WT, big-1, max4-1, and max4-1 big-1 at 5 DAG. Scale bar, 5 mm. (D) Primary root length of WT, big-1, max4-1, and max4-1 big-1 at 5 DAG. Values are means ± SD (n ≥ 30). One-way ANOVA, Tukey’s multiple comparisons test. Different letters indicate values are significantly different (p < 0.01). (E) Representative CLSM image of WT, big-1, max4-1, and max4-1 big-1 at 5 DAG. Scale bar, 50 µm. (F) Quantification of meristem cell number of WT, big-1, max4-1, and max4-1 big-1 seedlings at 5 DAG. Values are means ± SD (n ≥ 25). One-way ANOVA, Tukey’s multiple comparisons test. Different letters indicate values are significantly different (p < 0.01).

2.4. BIG Contributes to the SHR/SCR Pathway in Regulating Root Growth

Given that the SHR/SCR pathway is parallel to the PLT pathway in regulating root development [20], we then examined the possible relationship between BIG and SHR/SCR. For this purpose, shr-2 and scr-3 were crossed with big-1, respectively. As shown in Figure 5A–D, the lengths of both root and RAM of shr-2 big-1 and scr-3 big-1 double mutants were significantly shorter than big-1 but apparently similar to that of shr-2 or scr-3 single mutant, indicating that big-1 has no additive effects with shr-2 or scr-3 on the root growth. These data suggest that BIG regulates root development via SHR/SCR actions. Next, CLSM was used to observe the root cytological morphology of shr-2 big-1 and scr-3 big-1 double mutants. As shown in Figure 5D, the double mutants shr-2 big-1 and scr-3 big-1 showed the comparable phenotype of a single GT layer to either shr-2 or scr-3 single mutant. Notably, the SCN morphology of either the shr-2 big-1 or the scr-3 big-1 double mutants resembles that of shr-2 or scr-3 (Figure 5D), indicating BIG likely acts through the SHR/SCR pathway to regulate root patterning and growth. To evaluate whether mutations in the BIG gene affect the expression of the SHR gene, we crossed big-1 with the transgenic plants that express erGFP under the control of the SHR promoter. The fluorescence intensity of pSHR::erGFP [48] in big-1 was significantly lower than that of WT (Figure 6A,B), suggesting that BIG positively regulates the expression of the SHR gene. Likewise, to determine whether a big mutant affects SCR expression, we crossed the big-1 mutant with pSCR::GFP [10]. The fluorescence intensity of pSCR::GFP was substantially...
lower in the endodermis of big-1 than that in WT (Figure 6C,D), suggesting that BIG positively regulates the expression of SCR. RT-qPCR analysis showed that big mutant suppresses the expression of the SHR and SCR genes (Figure 6E). Taken together, these findings indicate that the BIG gene is involved in the SHR/SCR pathway that regulates root growth and patterning in Arabidopsis.

Figure 4. BIG and PLTs function in the independent pathway. (A) Images of the indicated genotypes WT, big-1, plt1-4 plt2-2, and plt1-4 plt2-2 big-1 seedlings at 5 DAG. Scale bar, 5 mm. (B) Primary root length of WT, big-1, plt1-4 plt2-2, and plt1-4 plt2-2 big-1 seedlings at 5 DAG. Values are means ± SD (n ≥ 30). One-way ANOVA, Tukey’s multiple comparisons test. Different letters indicate values are significantly different (p < 0.01). (C) Representative CLSM image of WT, big-1, plt1-4 plt2-2, and plt1-4 plt2-2 big-1 seedlings at 5 DAG. Scale bar, 25 µm. (D) Quantification of meristem cell number of WT, big-1, plt1-4 plt2-2, and plt1-4 plt2-2 big-1 seedlings at 5 DAG. Values are means ± SD (n ≥ 30). One-way ANOVA, Tukey’s multiple comparisons test. Different letters indicate values are significantly different (p < 0.01). (E,G) pPLT1:erCFP (E) and pPLT2:erCFP (G) expression in WT and big-1 seedlings at 5 DAG. Scale bars, 20 µm. (F,H) Quantification of pPLT1:erCFP (F) and pPLT2:erCFP (H) relative intensity in WT and big-1 seedlings at 5 DAG. Values are means ± SD (n ≥ 20). Student’s t-test. Different letters indicate values are significantly different (p < 0.05). (I) qRT-PCR analyses show the relative expression levels of PLT1 and PLT2 in WT and big-1 seedlings. Error bars represent SD. Two-way ANOVA, Tukey’s multiple comparisons test. Different letters indicate values are significantly different (p < 0.01).
Figure 5. BIG acts in the SHR/SCR pathway to regulate root growth. (A) Images of the indicated genotypes WT, big-1, scr-3, scr-3 big-1, shr-2, and shr-2 big-1 seedlings at 5 DAG. Scale bar, 5 mm. (B) Primary root length of WT, big-1, scr-3, scr-3 big-1, shr-2, and shr-2 big-1 seedlings at 5 DAG. Values are means ± SD (n ≥ 30). One-way ANOVA, Tukey’s multiple comparisons test. Different letters indicate values are significantly different (p < 0.01). (C) Quantification of meristem cell number of WT, big-1, scr-3, scr-3 big-1, shr-2, and shr-2 big-1 seedlings at 5 DAG. Values are means ± SD (n ≥ 30). One-way ANOVA, Tukey’s multiple comparisons test. Different letters indicate values are significantly different (p < 0.01). (D) Root apical phenotypes of WT, big-1, scr-3, scr-3 big-1, shr-2, and shr-2 big-1 at 5 DAG. The insets show root radial patterning surrounded by white rectangles. en—endodermis; co—cortex; ep—epidermis; m—mutant cell layer in shr-2 or scr-3. Scale bars, 50 µm.
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3. Discussion

Previous studies have shown the big mutants with shorter roots and reduced meristem length [28,29,35]. QC maintains the fate of stem cells and prevents them from differentiation, which is important for the development of roots; the QC-specified expressed WOX5 gene is indispensable to SCN maintenance [2,4,5]. The BIG gene is required to maintain QC activity; the expression of QC46 and WOX5 was decreased in the big mutant (Figure 1A–D); at the same time, the CSCs development in the big mutant was disturbed (Figure 1F). The wox5-1 big-1 double mutant showed a comparable QC phenotype to that of wox5-1 (Figure 1E), indicating that WOX5 has epistatic effects on BIG in maintaining SCN. These data indicate that the BIG gene is required for maintaining SCN integrity.

The BIG gene was originally identified in a genetic screen for mutants that are insensitive to the auxin transport inhibitor NPA and has been assigned a role in polar auxin transport and in auxin-inhibited endocytosis [28,29,32,34]. BIG acts synergistically with PIN1 to control the development of leaves and shoot apical meristems [35,49]. Auxin accumulation in root tips is reduced by a big-1 mutant (Figures 2A and 3A). Consistently, BIG gene deficiency resulted in a marked decrease in the expression of the PIN gene family (Figure 2B–J).
Our results suggest that BIG impacts polar auxin transport by modulating the expression of the PIN genes (Figure 2B–J). However, it is unclear how BIG regulates the expression of the PINs and consequently affects the polar transport of auxin in root tips. A possible explanation is that BIG regulates the expression of PINs through auxin-inhibited endocytosis, which in turn modulates the polar transport of auxin [34]. The max4-1 mutant has been used to efficiently increase the auxin transport activity but failed to alleviate the over-branched phenotype of the big mutant [42]. Neither increasing auxin transport capacity nor by increasing the auxin levels in big mutants could restore the RAM defects (Figure 3A–F and Figure S1B–F), suggesting that the impaired auxin transport capacity is not, at least not mainly, responsible for the root developmental defects in big mutants, in line with previous reports that mutations in the BIG gene hardly caused any abnormal responses to auxin applications [28,35].

Auxin and PLTs function together to render the feedback regulation in the root development system [20–23]. plt1-4 plt2-2 double mutants exhibit prematurely differentiated root meristems [20]. This study shows that the meristem exhaustion in the plt1-4 plt2-2 double mutant is faster than that in the big mutant background (Figure 4A–D), which suggests that BIG functions without relying on the PLT pathway in regulating the RAM activity and SCN integrity. However, disruption of BIG could suppress the expression of the PLTs genes (Figure 4E–I). This is likely due to the relatively lower auxin accumulation in the enlarged roots caused by the big mutant, which subsequently suppressed the auxin-induced PLTs expression (Figures 2A and 3A). Together, these findings strongly indicate that despite its involvement in auxin transport, BIG functions through a pathway independent of the PLT pathway in the regulation of root development.

SHR/SCR functions in parallel to the PLTs in regulating root development [20]. Our genetic investigations show that BIG deficiency has no additive effects with either shr-2 or scr-3 on root growth (Figure 5A–D). Given that mutations in either the SHR or SCR gene resulted in a single GT layer, whereas shr’s GT has cortex cell characteristics and scr’s GT has both cortex cell and endodermis cell characteristics, SHR has been ascribed an additional role in the endodermis cell fate specification [9–13]. The double mutants shr-2 big-1 and scr-3 big-1 showed similar phenotypes of a single GT layer, indicating that BIG regulates the development of GT via the SHR/SCR pathway (Figure 5D). Our data indicate that the shr-2 big-1 and scr-3 big-1 double mutants have similar QC-deficient morphology to either shr-2 or scr-3 (Figure 5D, [9–13]). These results suggest that BIG modulates root SCN via SCR/SHR pathway. Disrupting BIG will simultaneously reduce the expression of the SHR gene and the SCR gene (Figure 6A–E). As the only homolog of pRB in higher plants, RBR is highly conserved across species [50]. To govern cell adhesion in human cells, pRB and UBR4 interact directly and co-localize in the nucleus [24]. UBR4 is a UBR box E3 ligase that uses the N-degron pathway to degrade proteins [24–27]. Sequence similarity analysis reveals that BIG has the conserved UBR box and E3 ligase domains (Figure S2A), pointing to a scenario that BIG might also regulate RBR levels through the N-degron pathway. Taken together, we propose that BIG modulates meristem development and SCN integrity via the SCR/SHR pathway in Arabidopsis roots and the BIG-mediated polar auxin transport also contributes to this process (Figure 6F). Further investigation is required to experimentally establish a direct relationship between BIG and the SCR/SHR pathway in the future, though, considering the extra-large size of the putative BIG protein, it would be technically challenging.

4. Materials and Methods

4.1. Plant Material and Growth Conditions

Arabidopsis thaliana ecotype Columbia (Col) was used as WT. Seeds of big-2 (CS903939), shr-2 (N2972), and scr-3 (N3997) were obtained from NASC (the European Arabidopsis Stock Centre, http://arabidopsis.org.uk (accessed on January 2018)). Other mutant and transgenic plant lines used in this study including big-1 [36], QC46 [5], pWOX5:erGFP [39], wox5-
1 [4], pDR5::GUS [40], pPIN1::PIN1::GFP [41], pPIN2::PIN2::GFP [16], pPIN3::PIN3::GFP [16], pPIN7::PIN7::GFP [16], pDR5rev::GFP [43], max4-1 [42], tir3-101 [42], YUCCA1-OX [47], plt1-4 plt2-2 [20], pPLT1::erCFP [23], pPLT2::erCFP [23], pSHR::erGFP [48], and pSCR::GFP [10] were kind gifts from the authors.

After surface-sterilizing for 10 min in 70% ethanol, the seeds were placed on sterilized filter paper in a laminar air flow hood and blown dry before sowing on a half-strength Murashige and Skoog (1/2 MS, Sigma, St. Louis, MO, USA) media plate [51] with 0.8% agar (Sigma) and 1% sucrose. Next, the seed plates were placed at 4 °C for no less than 2 days in the dark and then vertically placed into a plant greenhouse at 22–23 °C with a 16 h light (light intensity: 120 µmol photons m⁻²s⁻¹)/8 h dark photoperiod. If not stated otherwise, Arabidopsis roots were investigated at 5 DAG.

4.2. Histology and Microscopy

The GUS histochemical staining was performed as previously described [35] with minor modifications. Briefly, the seedlings were fixed using 90% acetone for 20 min; adding 1 mL of GUS lotion (pH 7.0, 2 mM ferricyanide, 2 mM ferrocyanide, and 10 mM Ethylene Diamine Tetraacetic Acid) to wash twice to remove the acetone; adding GUS staining solution (GUS lotion with 1 mM X-glucuronide), vacuuming on ice for 15–20 min; placing in the dark at 37 °C for an appropriate time according to the experimental conditions; agitating the staining solution, adding 0.8 mL 70% ethanol to stop the staining reaction and decolorization; changing the ethanol several times until the decolorization is complete. An Olympus IX70 microscope (Olympus, Tokyo, Japan) was used to capture the differential interference contrast images and image processing with Olympus cellSens software (1.6, Olympus, Tokyo, Japan).

The mPS-PI staining was performed essentially as previously described [52]. Arabidopsis seedlings were fixed in 2% formaldehyde solution for 25–30 min. The fixed sample was soaked in methanol at room temperature for 15 min, rinsed for 5 min, then placed in a 1% periodic acid solution for 25–30 min at 22–25 °C. Samples were then rinsed for 5 min, treated with Schiff reagent (1 mM sodium thiosulfate, 0.15 M HCl), 5 µg/mL propidium iodide (PI, Sigma) added, and stained for 15–30 min.

For CLSM, the roots of 4–6 DAG Arabidopsis seedlings were incubated in PI (10 µg/mL) for 3–5 min, using a Leica SP8 system (Leica, Wetzlar, German) to observe. Fluorescence of GFP, CFP, YFP, and PI staining was visualized using the settings: excitation wavelength 488 nm and emission wavelength from 505 to 550 nm for GFP, excitation wavelength 458 nm and emission wavelength from 463 to 500 nm for CFP, and excitation wavelength 561 nm and emission wavelength from 600 to 650 nm for PI staining, respectively. Leica AF Lite software (3.3.10, Leica, Wetzlar, German) was used to capture the images. Three biological replicates were generated. We quantified fluorescence intensity using 20–30 roots with ImageJ (1.8.0, National Institutes of Health, USA). The same microscope settings were used to observe WT and big seedlings. One-way ANOVA (Tukey’s multiple comparisons test) or Student’s t-test was used for significance analysis.

4.3. The Reverse Transcription-Quantitative PCR Assays

Approximately 5–10 mm of 5 DAG seedlings’ apical parts were used to extract RNA. cDNA was prepared by PrimeScript™ RT reagent Kit (TaKaRa, Bio Inc. Shiga, Japan) and quantified on a Bio-Rad CFX384 Touch fluorescence quantitative PCR instrument (Bio-Rad, Hercules, CA, USA) with the SYBR® Green Realtime PCR Master Mix (TOYOBO, Tokyo, Japan). The Actin7 gene was used as an internal reference gene. Three biological replicates were generated. One-way ANOVA (Tukey’s multiple comparisons test) or Student’s t-test was used for significance analysis. The primers used are listed in Table S1.
4.4. Accession Number

AT3G02260 (BIG), AT3g11260 (WOX5), AT4G32810 (MAX4), AT4G32540 (YUCCA1), AT1G73590 (PIN1), AT5G57090 (PIN2), AT1G70940 (PIN3), AT1G23080 (PIN7), AT3g54220 (SCR), AT4G37650 (SHR), At3g20840 (PLT1), At1g51190 (PLT2).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23126784/s1.

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