JIPB Journal of Integrative Plant Biology

ARF2 coordinates with PLETHORAs and PINs to orchestrate ABA-mediated root meristem activity in *Arabidopsis*[FA]

High-Impact Article

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doi: 10.1111/jipb.12506

Abstract Multiple hormones, including abscisic acid (ABA) and auxin, regulate cell division and differentiation of *Arabidopsis* root meristems. AUXIN RESPONSE FACTOR 2 (ARF2) functions as a negative regulator of ABA responses, as seed germination and primary root growth of *arf2* mutants are hypersensitive to ABA. In this study, we found that ABA treatment reduced the expression levels of the PIN-FORMEDs (PIN) auxin efflux carriers, PIN1, PIN3, PIN4, and PIN7, to a greater extent in the root meristems of *arf2-101* mutant than in the wild type. Also, *arf2-101 pin1* and *arf2-101 pin4* double mutants show less ABA-induced inhibition of root meristem activity than the *arf2-101* mutants. Furthermore, ARF2 positively mediates the transcripts of transcription factor *PLETHORA 1* (*PLT1*) gene but negatively mediates PLT2 at protein level in root meristems. Using a dexamethasone (DEX)-inducible transgenic line, *Pro35S:PLT2-GR*, we showed that PLT2 greatly promotes cell division and completely inhibits cell differentiation in root meristems of the *arf2-101* mutant once PLT2 is induced by DEX, which can be partially reversed by ABA treatment, suggesting that ABA regulates root meristem activity in both ARF2-dependent and independent pathways. Our results uncover a complex regulatory architecture in which ARF2 coordinates with PLTs and PINs to orchestrate ABA-mediated regulation of root meristem activity in *Arabidopsis*.

Edited by: Alice Y. Cheung, University of Massachusetts, USA
Received Oct. 7, 2016; **Accepted** Nov. 1, 2016; **Online on** Nov. 7, 2016
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INTRODUCTION

Root growth is dynamically regulated by a network of interacting hormone signals that coordinate cell division and differentiation to maintain the root apical meristem (RAM) (Lee et al. 2013). Water stress induces the accumulation of ABA, which can promote primary root growth at low concentrations and inhibit primary root growth at high concentrations (Zhang et al. 2010). Some DNA replication-related mutants have small RAMs and are hypersensitive to ABA, suggesting that ABA inhibits cell division through DNA replication-related proteins (Yin et al. 2009; Yao et al. 2013). Previous studies indicated that ABA signaling could promote ethylene production via phosphorylation of the C-termini of 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE (ACS), and stabilization of ACSs by ABA-activated calcium-dependent protein kinase 4 (CPK4) and CPK11 (Luo et al. 2014). Ethylene signaling upregulates WEAK ETHYLENE INSENSITIVE2 (WEI2)/ANTHRANILATE SYNTHASE alpha1 and WEI7/ /ANTHRANILATE SYNTHASE beta1 to synthesize auxin and inhibit primary root growth (Stepanova et al. 2005; Mao et al. 2016). ABA also promotes the production of reactive oxygen species in mitochondria, reduces auxin accumulation, and results in the inhibition of primary root growth (He et al. 2012; Yang et al. 2014).

Auxin plays important roles in RAM establishment and maintenance (Overvoorde et al. 2010). The PIN-

FORMEDs (PINs) polar auxin efflux transporters are critical for producing a proper auxin gradient in RAMs (Blilou et al. 2005; Petrasek and Friml 2009). In the primary root, basally localized PIN1, PIN3, and PIN7 in the stele mediate auxin flow towards the tip, which leads to high concentrations of auxin in the stem cell niche. PIN4, PIN3, and PIN7 expressed in the columella cells distribute auxin to the root cap and the epidermis, where the apically localized PIN2 mediates the upward flow of auxin to the elongation zone (Friml et al. 2002; Friml et al. 2003; Friml et al. 2004; Blilou et al. 2005; Petrasek and Friml 2009). In RAMs, key transcription factors such as WUSCHEL-RELATED HOMEOBOX 5, PLETHORA (PLT), SHORT ROOT, and SCARECROW, which respond to intercellular signaling, ultimately regulate cell division and differentiation (DiLaurenzio et al. 1996; Helariutta et al. 2000; Sabatini et al. 2003; Aida et al. 2004; Blilou et al. 2005; Sarkar et al. 2007). Recent studies indicated that the levels of PLT proteins play crucial roles in determining cell fate in the RAM (Galinha et al. 2007; Mahonen et al. 2014). A PIN-mediated auxin gradient underlies the PLT gradients and the PLT gradients in turn affect PIN levels (Friml et al. 2003; Blilou et al. 2005; Galinha et al. 2007; Mahonen et al. 2014). However, prolonged high auxin levels promote a narrow domain of *PLT* transcription from which a PLT gradient is produced to define the developmental zones (Mahonen et al. 2014). The expression of *PLT*s is modulated by different factors, including the auxin response factors MONOPTEROS (MP), NON-PHOTO-TROPIC HYPOCOTYL4 (NPH4) (Aida et al. 2004), MYC2 (Chen et al. 2011b), and RopGEF7 (Chen et al. 2011a), and receptor-like protein kinases RGFR1/2/3 (Shinohara et al. 2016), and a SWI/SNF chromatin remodeling ATPase BRAHMA (Yang et al. 2015). High PLT levels in root stem cell niches promote stem cell identity and maintenance, moderate PLT levels promote stem cell division, and low PLT levels are required for cell differentiation (Galinha et al. 2007; Mahonen et al. 2014).

Previous studies indicated that ARF2 has multiple roles in regulating plant development and responses to different hormones (Li et al. 2004; Okushima et al. 2005; Schruff et al. 2006; Vert et al. 2008; Wang et al. 2011). ARF2 inhibits the expression of the homeodomain gene *HB33*, which mediates the ABA response in *Arabidopsis* (Wang et al. 2011). The root growth of the *arf2* mutants is hypersensitive to ABA, but this sensitivity can be rescued by the addition of the auxin influx inhibitor 1-naphthoxyacetic acid (Wang et al. 2011). These results suggest that ABA inhibition of root growth in the *arf2* mutant involves auxin signaling (Wang et al. 2011). In this study, we further explored the mechanism for ARF2 regulation of root growth in ABA signaling. We found that the *arf2* mutant changes the expression of PINs, PLT1, and PLT2. Interestingly, dexamethasone (DEX)-induced PLT2-GR in the *Pro35S:PLT2-GR* transgenic line triggers increased cell division but completely blocks cell differentiation in the RAM of the *arf2* mutant, and this could be partially inhibited by ABA treatment. Our results thus uncover an important interplay among ARF2, PINs, and PLT2 in ABA-mediated root meristem activity.

RESULTS

ARF2 regulates RAM activity and cell elongation in ABA signaling

A previous study indicated that ARF2 regulates root growth through ABA signaling (Wang et al. 2011). The *arf2-101* mutant showed enhanced ABA sensitivity in seed germination and primary root growth (Wang et al. 2011). Under normal conditions, the root meristem zone and elongation zone were slightly smaller in the *arf2-101* mutant than in the wild type (WT) (Figure 1A–C). Under ABA treatment, the length of the meristem zone (MZ) and cell number in the MZ were significantly reduced in the *arf2-101* mutant, but the average cell length in the MZ did not change much when compared with the WT (Figure 1A–C). The length of the elongation zone (EZ) and cell length in the EZ, but not cell number, were all significantly reduced in the *arf2-101* mutant after ABA treatment, compared with the WT (Figure 1A, B, D). These results indicate that ARF2 positively regulates RAM activity in ABA signaling.

ARF2 alters the accumulation of PIN proteins in the RAM after ABA treatment

A previous study showed that the expression of PIN3 and PIN7 decreased in the *arf2-101* mutant under ABA treatment, compared with the WT (Wang et al. 2011). In this study, we examined the expression of *PIN*s by crossing the *arf2-101* mutant with constructs encoding green fluorescent protein (GFP) fusions to the PIN proteins, expressed under the control of their

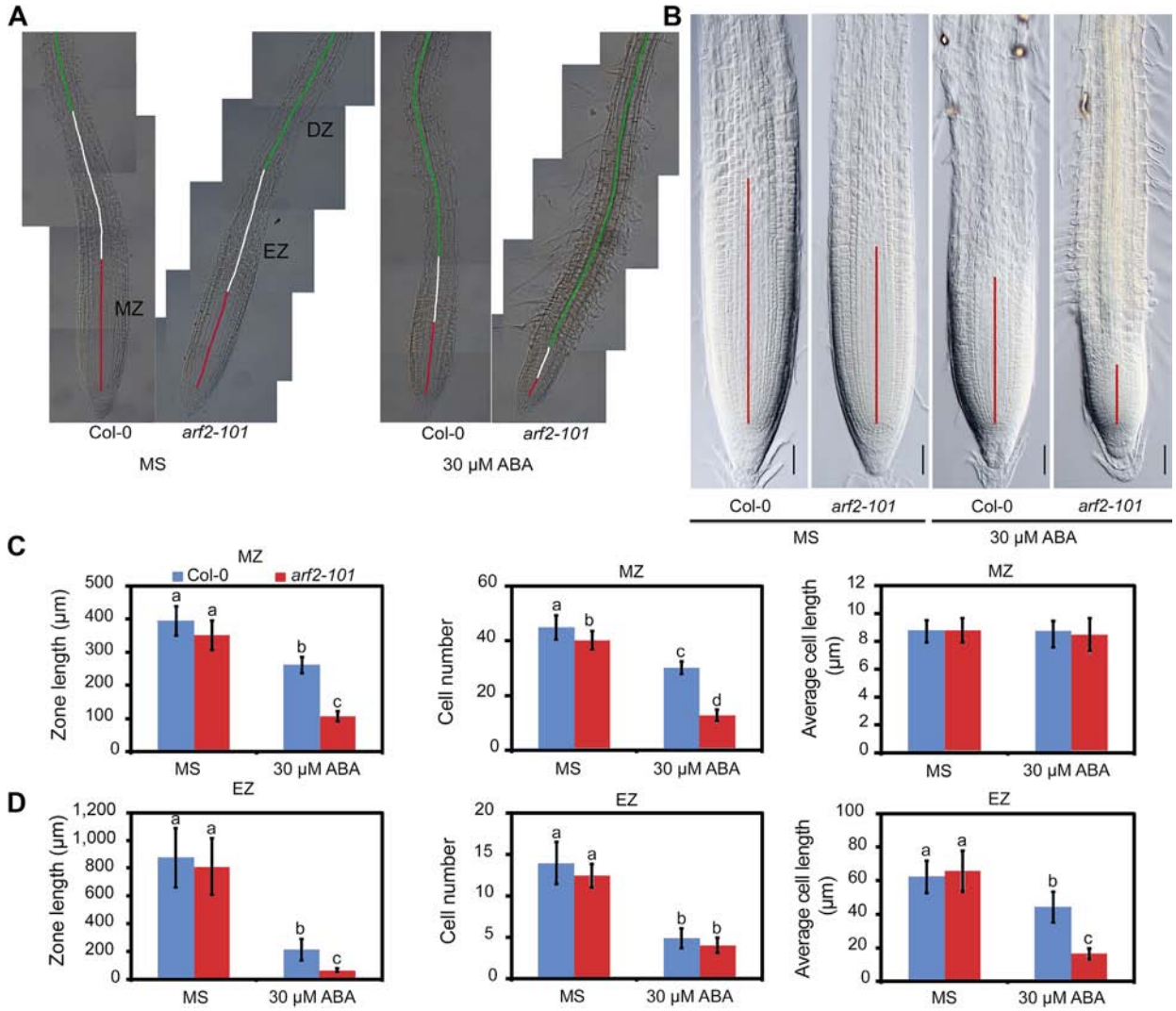

Figure 1. Abscisic acid (ABA) treatment inhibits the meristem and elongation zones in Col-0 and the *arf2-101* mutant

(A) Comparison of root growth in the wild type (WT) and *arf2-101* mutants. Four-day-old seedlings grown on MS medium were transferred onto MS medium (control) or MS medium with 30 μM ABA for 3 d. The meristem zone (MZ): red line; elongation zone (EZ): white line; and differentiation zone (DZ): green line. Scale bars = 100 μm. Each image was made by joining several photographs of the same root. **(B)** A closer look at the root meristem phenotypes of Col-0 and the *arf2-101* mutant seedlings in (A). The meristem zone is marked with a red line. Bars = 50 μm. **(C)** The root meristem zone (MZ) length, cell number, and average cell length of the WT and the *arf2-101* mutant in **(A)**. **(D)** The root elongation zone (EZ) length, cell number and average cell length of the WT and the *arf2-101* mutant **(A)**. Three independent experiments were conducted with similar results in **(B)** and **(C)**. Each experiment was conducted with 12-25 roots from three plates. Values are means ± *SD*. Means with different letters are significantly different at $P < 0.01$.

native promoters. We found that the levels of PIN1-GFP, PIN3-GFP, and PIN7-GFP in the root tips of the *arf2-101* plants with the reporter constructs were higher than those in the WT grown on MS medium, and the expression of PIN4-GFP significantly increased in the *ProPIN4:PIN4-GFP arf2-101* transgenic plants relative to the WT (Figure 2A–H). ABA treatment decreased the signals for all PINs in the *arf2-101* transgenic plants and in the WT, but the GFP signals decreased more in *arf2-101* than in WT (Figure 2A–H). We also noted that after ABA treatment, the PIN4-GFP signal was hardly detected in the WT, but could be

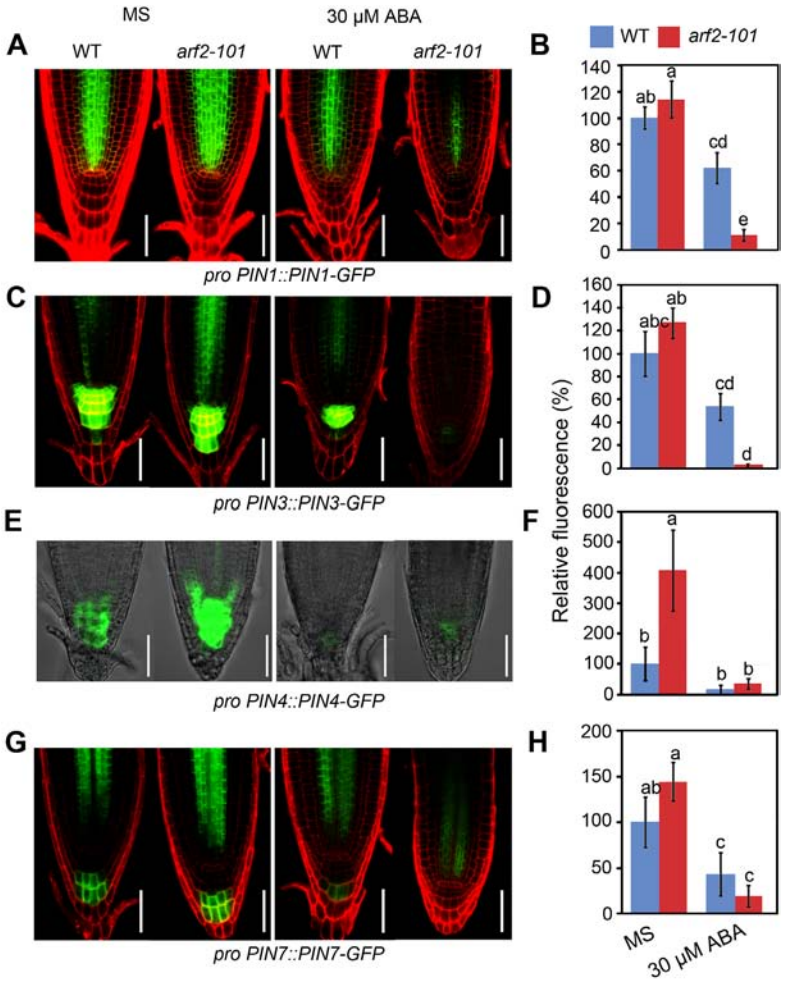

Figure 2. The Expression of PIN1-, PIN3-, PIN4-, and PIN7-GFP in primary root tips of the *arf2-101* mutant
Fluorescence images and the relative fluorescence of *ProPIN1:PIN1-GFP* **(A** and **B)**, *ProPIN3:PIN3-GFP* **(C** and **D)**, *ProPIN4:PIN4-GFP* (**E** and **F**), and *ProPIN7:PIN7-GFP* **(G** and **H)** in the root tips of the wild type (Col-0) and the *arf2-101* mutant. Four-day-old seedlings were transferred onto MS medium or MS medium supplemented with 30 μM abscisic acid (ABA) for 2 d. Three experiments were conducted with similar results. Each experiment was conducted with 8-15 roots from three plates. Values are means ± *SD*. $P < 0.01$. Means with different letters are significantly different at $P < 0.01$. Scale bar = 50 μm.

clearly observed in the *ProPIN4:PIN4-GFP arf2-101* transgenic plants. These results indicate that ARF2 mediates the expression of PINs in the RAM through ABA signaling.

PIN1 and PIN4 negatively mediate RAM activity of the *arf2-101* mutant in ABA signaling

When we introduced the *pin1-1*, *pin4-3*, *pin3-4*, and *pin7-2* mutations into the *arf2-101* mutant, we found that *pin1-1* and *pin4-3* could partially suppress the ABA-sensitive phenotype of the *arf2-101* mutant, but *pin3-4* and *pin7-2* could not (Figures S1–S4), and *pin1-1* conferred slightly more sensitivity to ABA than the WT. The *arf2-101 pin1-1* double mutant had a smaller RAM than the *arf2-101* or *pin1-1* single mutants under normal growth conditions (Figure 3A–C). Under a 10 or 30 μM ABA treatment, the *arf2-101 pin1-1* double mutant had a larger RAM and longer cells in the Elongation zone (EZ) than the *arf2-101* mutant (Figure 3A–C). PIN4 is associated with root meristem activity and patterning (Friml et al. 2002; Garay-Arroyo et al. 2013). In both the control and in the ABA treatment, the *arf2-101 pin4-3* double mutant had a larger meristem than the *arf2-101* mutant (Figure 4A). The *arf2-101 pin4-3* double mutant had more cell number and longer zone length than *afr2-101* in

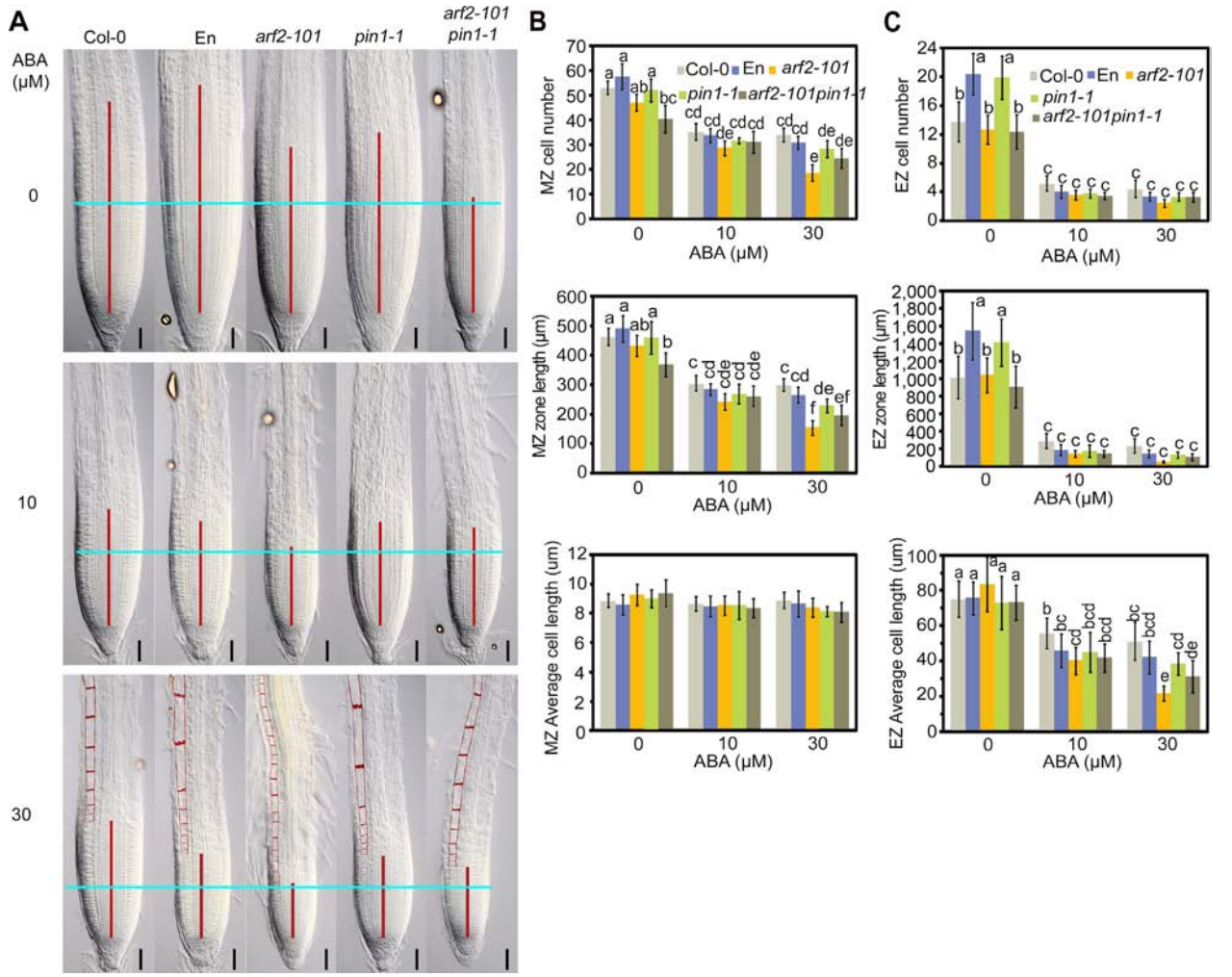

Figure 3. The *pin1* mutation reduces ABA-mediated inhibition of RAM activity in the *arf2-101* mutant
(A) The primary root meristem of the Col-0 and En ecotypes, the *arf2-101*, *pin1-1* (En), and *arf2-101 pin1-1* mutant seedlings on MS medium with and without ABA. Five-day-old seedlings were transferred to MS medium or MS supplemented with different concentrations of ABA for 5 d before taking images. The meristem zone is marked with a red line. The epidermal cell shapes are outlined in red in the 30 μM ABA treatment for comparison of cell size. Bars = 50 μm. **(B)** The root meristem zone (MZ) cell number, zone length and average cell length of each ecotype **(A)**. **(C)** The root elongation zone (EZ) cell number, zone length and average cell length of each ecotype in **(A)**. Three independent experiments were conducted with similar results in **(B)** and **(C)**. Each experiment was conducted with 12–25 roots from three plates. Values are means ± *SD*. Means with different letters are significantly different at $P < 0.01$.

meristem zone (Figure 4B), and longer cell length in EZ (Figure 4C) under ABA treatment. These results suggest that PIN1 and PIN4 negatively regulate RAM activity of the *arf2-101* mutant under ABA signaling.

ARF2 affects the expression of PLT1 and PLT2 in root meristems

The *PLETHORA* genes have crucial functions in root patterning and auxin readout mechanisms, and the transcriptional gradients of *PLT1* and *PLT2* strongly correlate with the auxin gradients in the RAM (Aida et al. 2004; Galinha et al. 2007). Transcription of *PLT* genes is stimulated by auxin and is dependent on ARF transcription factors such as MONOPTEROS/ARF5 and NONPHOTOTROPIC HYPOCOTYL 4/ARF7 (Aida et al. 2004). To test whether ARF2 has any effect on the expression of *PLT1* and *PLT2* in root apical meristems, we crossed the

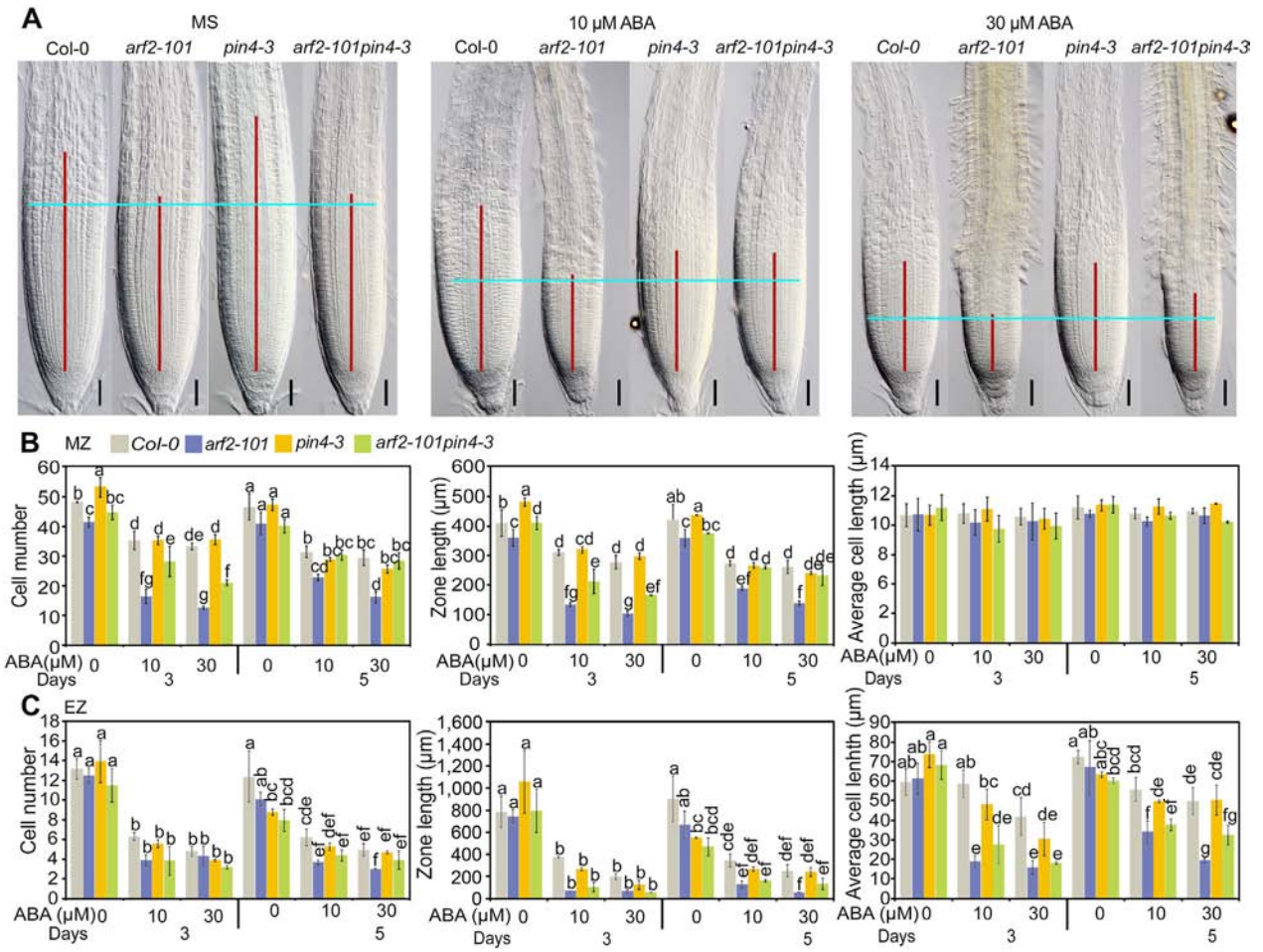

Figure 4. The *pin4* mutation reduces abscisic acid (ABA)-mediated inhibition of RAM activity in the *arf2-101* mutant
(A) The root meristem phenotype of Col-0 and the *arf2-101*, *pin4-3* and *arf2-101 pin4-3* mutants after 5-day-old seedlings were transferred onto MS medium or MS medium supplemented with 30 μM ABA for 5 d. The meristem zone is marked with a red line. Bars = 50 μm. **(B)** The root meristem zone (MZ) cell number, zone length, and average cell length of each ecotype in **(A)**. **(C)** The root elongation zone (EZ) cell number, zone length and average cell length of each ecotype in **(A)**. Three independent experiments were conducted with similar results in **(B)** and **(C)**. Each experiment was conducted with 12−25 roots from three plates. Values are means ± *SD*. Means with different letters are significantly different at $P < 0.01$.

arf2-101 mutant with the transgenic plants carrying *ProPLT1:PLT1-YFP*, *ProPLT2:PLT2-YFP*, *ProPLT1:CFP* or *ProPLT2:CFP*. The expression of *ProPLT1:PLT1-YFP* and *ProPLT1:CFP* was lower in the *arf2-101* transgenic plants than in the corresponding transgenic WT (Figure 5A, C, E, G). ABA treatment reduced the expression of *ProPLT1:PLT1-YFP* and *ProPLT1:CFP* in both the *arf2*-101 transgenic plants and in the corresponding transgenic WT (Figure 5A, B, E, F). These results suggest that ARF2 positively regulates the expression of *PLT1*.

The expression of *PLT2-YFP* was slightly reduced in the RAM of the *ProPLT2:PLT2-YFP arf2-101* transgenic plants compared with the *ProPLT2:PLT2-YFP*/Col-0 root under normal conditions (Figure 5C, D). ABA treatment at different concentrations reduced the expression of *ProPLT2:PLT2-YFP* in the WT root, but had no effect on its accumulation of PLT2-YFP in the *ProPLT2:PLT2-YFP arf2-101* plants (Figure 5C, D). However, *ProPLT2:CFP* expression was lower in the *ProPLT2:CFP arf2-101* plants than in the *ProPLT2:CFP*/Col-0 plants under normal conditions, and was reduced by ABA treatment to a similar level in both plants (Figure 5G, H). These results suggest that ARF2 negatively regulates PLT2 at the post-transcriptional level in ABA signaling.

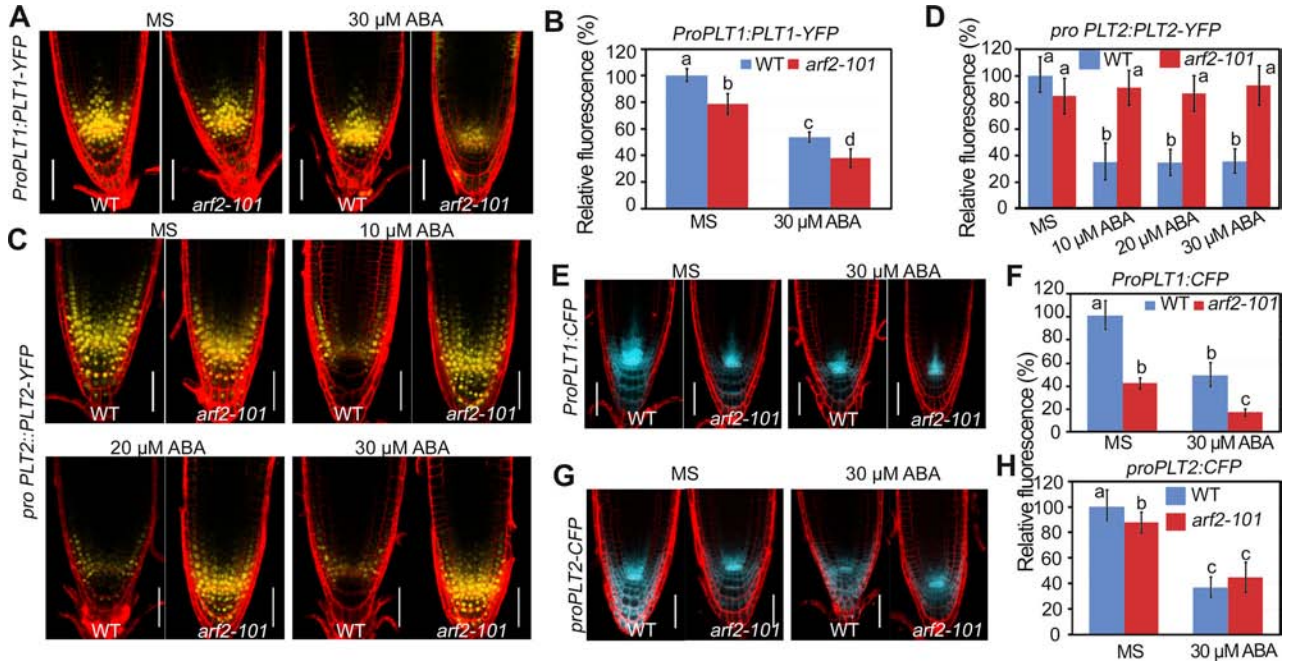

Figure 5. ARF2 affects the expression of *PLT1* and *PLT2*

(A, B, E, F) The expression of *ProPLT1:PLT1-YFP* (A) and *ProPLT1:CFP* **(E)** in the *arf2-101* mutant and the WT. Four-day-old seedlings were transferred onto MS medium or MS medium supplemented with 30 μM ABA for 2 d. Scale bar = 50 μm. The relative fluorescence of *ProPLT1:PLT1-YFP* **(B)** in **(A)** and *ProPLT1:CFP* (F) in **(E)**. Each experiment was conducted with 8-15 roots. Values are means ± SD. Means with different letters are significantly different at $P < 0.01$. **(C, D, G, H)** The expression of *ProPLT2:PLT2-YFP* (**C**) and *ProPLT2:CFP* (**G**). Four-day-old seedlings were treated with 0, 10, 20, and 30 μM ABA for 2 d. Scale bar = 50 μm. The relative fluorescence of *ProPLT2:PLT2-YFP* **(D)** in **(C)** and *ProPLT2:CFP* **(H)** in **(G)**. Each experiment was conducted with 8-15 roots. Values are means ± *SD*. Means with different letters are significantly different at $P < 0.01$.

Overexpression of *PLT2* promotes cell division and prevents cell differentiation in the *arf2-101* mutant

Although PLTs are functionally redundant and their activities in regulating the cell fate of the RAM are largely additive and dosage dependent, the different modes by which AFR2 modulates PLT1 and PLT2 suggest that PLT1 and PLT2 may have some independent roles in mediating root growth in ABA signaling. As PLT2 is likely regulated by ARF2 at the post-transcriptional level, to study the effect of PLT2 on root growth, we introduced a DEX-inducible PLT2-GR into the *arf2-101* mutant by crossing a *Pro35S:PLT2-Glucocorticoid Receptor* (*Pro35S:PLT2-GR*) transgenic plant with the *arf2-101* mutant. We observed root meristem patterns directly after seed germination and after the 4-day-old seedlings were transferred onto DEX containing medium for 3 d. The root meristem cell number of the *Pro35S:PLT2-GR*/Col-0 plants increased from 38, with no DEX induction, to 43 after DEX treatment (Figure 6A–C). Surprisingly, the root meristem cell number of the *Pro35S:PLT2-GR/arf2-101* transgenic plants increased from 43, with no DEX induction, to 173 after DEX treatment (Figure 6A–C). Similarly, the root meristem cell number of the *Pro35S:PLT2-GR*/Col-0 plants increased from 28, with no DEX induction, to 35 after DEX treatment, and from 31, with no DEX induction, to 157 after DEX treatment in the *Pro35S:PLT2-GR/arf2-101* transgenic plants after seed germination for 6 d (Figure S5). The root meristem growth patterns indicated that once PLT2 is induced by DEX, it promotes cell division and at the same time completely blocks cell differentiation in the *arf2-101* mutant. These results suggest that ARF2 has an antagonistic effect on PLT2 in modulating cell division and differentiation and PLT2 acts downstream of ARF2.

We further examined the effect of ABA on root meristem cell number in these DEX-inducible lines. According to the sensitivity of seed germination and root growth to ABA, we used two concentrations of ABA: 30 μM for root growth and 0.3 μM for seed

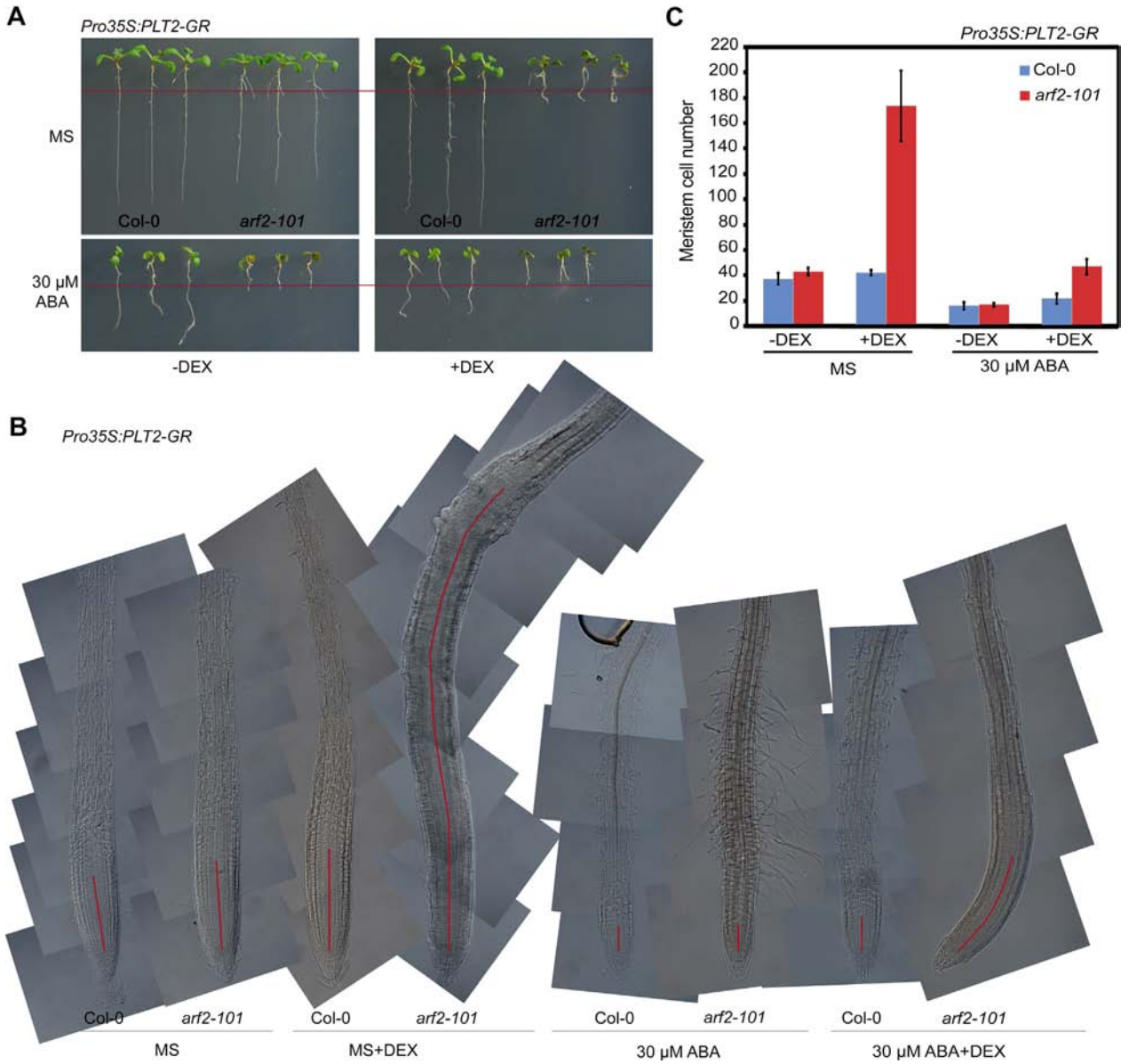

Figure 6. Overexpression of PLT2 in the *arf2-101* mutant promotes root meristem cell division and inhibits cell differentiation

(A) Seedling growth phenotypes after abscisic acid (ABA) and dexamethasone (DEX) treatment. Four-day-old seedlings were transferred to MS medium or MS medium supplemented with 2 μM DEX, 30 μM ABA or 30 μM ABA plus 2 μM DEX for 3 d. **(B)** Comparison of root meristems of *Pro35S:PLT2-GR/Col-0* and *Pro35S:PLT2-GR/arf2-101* seedlings in **(A)**. The meristem zone is marked with a red line. Scale bars = 50 μm. **(C)** Statistical analyses of root meristem number in **(B)**. Three experiments were conducted with similar results. Each experiment was conducted with 12-25 roots. Values are means ± *SD*.

germination. Without DEX induction, ABA treatment decreased the root meristem cell number of the seedlings from 38 to 16 in the *Pro35S:PLT2-GR*/Col-0 plants and from 43 to 17 in the *Pro35S:PLT2-GR/arf2-101* transgenic plants, and from 42 to 22 in the *Pro35S:PLT2-GR*/Col-0 plants and from 173 to 47 in the *Pro35S:PLT2-GR/arf2-101* transgenic plants with DEX induction (Figure 6A–C). Without DEX induction, ABA treatment decreased the root meristem cell number from 28 to 18 in the *Pro35S:PLT2-GR*/Col-0 plants and from 31 to 23 in the *Pro35S:PLT2-GR/arf2-101* transgenic plants after seed germination for 6 d (Figure S5). With

DEX induction, ABA treatment decreased the root meristem cell number from 35 to 22 in the *Pro35S:PLT2-GR*/Col-0 plants and from 157 to 49 in the *Pro35S:PLT2-GR/arf2-101* transgenic plants after seed germination for 6 d (Figure S5). These results suggest that the high induction of cell division by DEX-induced PLT2-GR in the *arf2-101* mutant could be partially inhibited by ABA treatment, and ABA inhibits cell division in both ARF2-dependent and −independent manners.

ARF2 mediates meristem cell division in parallel with PLT1, but not with PLT2

ARF2, PLT1, and PLT2 are negative regulators in ABA-mediated root growth (Wang et al. 2011; Yang et al. 2014). A previous study indicated that both the *plt1* and *plt2* single mutants display a slight but significant reduction in the growth rate and in the number of meristematic cells (Aida et al. 2004). To examine the genetic relationship between *ARF2* and *PLT1/2* in regulating meristem cell division, we generated double mutants by crossing *arf2-101* with *plt1-4* and *plt2-2* and observed meristem size. The root meristems of the *arf2-101 plt1-4* double mutants were smaller than those of the *arf2-101* and *plt1-4* single mutants on the control medium, suggesting that ARF2 and PLT1 have additive functions in mediating root meristem size (Figure 7A, B). The root meristem size of the *arf2-101plt2-2* double mutant was similar to that of the *plt2-2* mutant and slightly smaller than that of the *arf2-101* mutant. Under ABA treatment, the meristem size of the *arf2-101 plt1-4* double mutant was much smaller than either the *arf2-101* or *plt1-4* single mutants, further indicating that ARF2 and PLT1 act in a parallel manner to mediate root meristem size under ABA signaling. In contrast, the root meristem size of the *arf2-101 plt2-2* double mutant under ABA treatment was similar to that of the *arf2-101* mutant (Figure 7A, B), suggesting that a mutation in *PLT2* does not further enhance the ABA sensitivity of the *arf2-101* mutant in root growth. These results suggest that ARF2 acts synergistically with PLT1, and ARF2 and PLT2 do not have an additive effect on ABA-mediated root meristem activity.

DISCUSSION

Plant root growth is regulated by different hormones that have complex crosstalk with auxin. The auxin gradient in the root tip plays an important role in regulating root meristem division, differentiation, and root elongation (Aida et al. 2004; Galinha et al. 2007; Mahonen et al. 2014). Five auxin efflux transporter *PIN* genes (*PIN1*, *2*, *3*, *4* and *7*) are expressed in the roots of *Arabidopsis*. The PIN-dependent auxin distribution network involves redundancy and auxin-mediated cross-regulation of PIN expression and PIN targeting (Blilou et al. 2005; Vieten et al. 2005). ABA treatment inhibits both RAM activity and cell elongation in the elongation zone. Interestingly, we found that the expression of *PIN1*, *PIN2*, *PIN3*, *PIN4*, and *PIN7* genes was higher in the *arf2-101* mutant than in the WT under normal conditions. ABA treatment reduced their expression to a greater extent in the *arf2-101* mutant than in the WT. Both the *pin1* and *pin4* mutations increased RAM activity in the *arf2-101* mutant under ABA treatment, suggesting that PIN1 and PIN4 negatively mediate RAM activity in the *arf2-101* mutant under ABA signaling. In contrast, the *pin3* and *pin7* mutations did not exert an effect on RAM activity in the *arf2-101* mutant. PIN1 is mainly expressed in the vascular cylinder tissues, whereas PIN4 expression is localized in the quiescent center and surrounding cells of the root meristem (Friml et al. 2004; Blilou et al. 2005; Petrasek and Friml 2009). A recent study indicated that auxin concentrations in tissues is regulated by nonpolar AUX1/LIKE-AUXIN influx carriers, whereas the direction of auxin transport within the tissue is controlled by the polar transporter PINs (Band et al. 2014). Under ABA treatment, *ProIAA2:GUS* (an endogenous auxin marker) staining is lower in the *arf2-101* mutant than in the WT, suggesting that the *arf2-101* mutant roots might accumulate less auxin (Wang et al. 2011). However, the *aux1* mutation is able to completely reverse the hyper-sensitivity to ABA of the *arf2-101* mutant (Wang et al. 2011), while the *aux1* mutant accumulates less auxin in the root tips than the wild type (Swarup et al. 2001). We also found that root growth in the *pin1* mutant is more sensitive to ABA than in the WT because the *pin1* mutation did not enhance, but partially alleviated the ABA sensitivity of the *arf2-101* mutant. These results suggest that the ABA sensitivity in the roots of the *arf2* mutant is most likely due to auxin distribution and polar transporting.

Besides the PIN proteins, different transcription factors, such as PLT1, PLT2, PLT3, and PLT4, act downstream of auxin signaling in roots to regulate

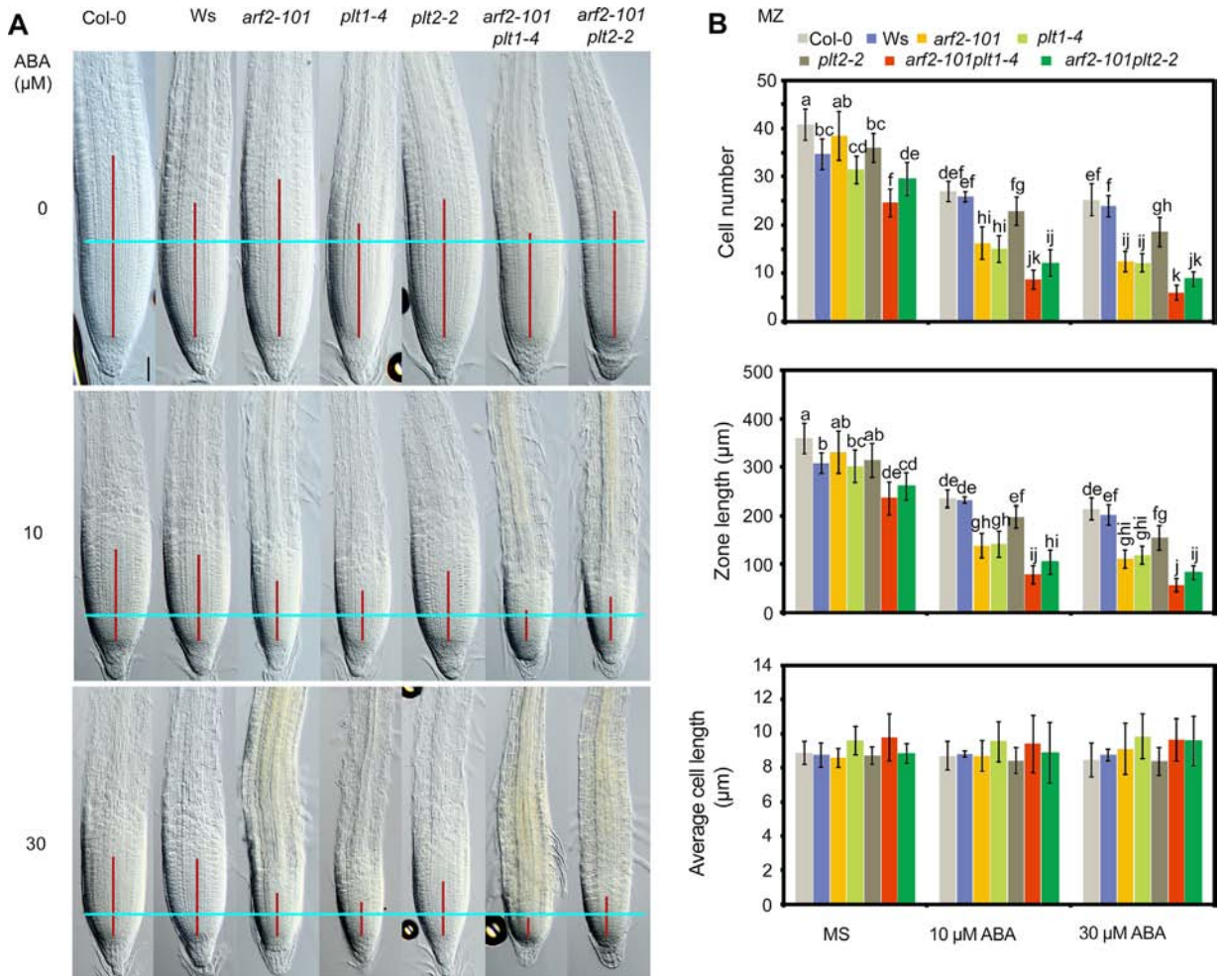

Figure 7. The *plt1-4* mutation, but not the *plt2-2* mutation, enhances the ABA-mediated inhibition of the root meristem in the *arf2-101* mutant

(A) The root meristem phenotypes of the Col-0 and Ws ecotypes and the *arf2-101*, *plt1-4*, *plt2-2* (WS), *arf2-101plt1-4* and *arf2-101plt2-2* mutants. Five-day-old seedlings were transferred onto MS medium or MS medium supplemented with 0, 10 or 30 μM ABA for 5 d. The meristem zone is marked with a red line. Bars = 50 μm. **(B)** The root meristem zone (MZ) cell number, zone length, and average cell length of each ecotype in **(A)**. Three independent experiments were conducted with similar results. Each experiment was conducted with 12–25 roots from three plates. Values are means ± *SD*. Means with different letters are significantly different at $P < 0.01$.

stem cell fate and cell division (Aida et al. 2004; Galinha et al. 2007; Mahonen et al. 2014). In this study, we found that the expression of *PLT1* at both the transcriptional and translational level was lower in the *arf2-101* mutant than in the WT with and without ABA treatment, suggesting that *PLT1* expression is affected by ARF2. However, our results demonstrated that the *plt1* and *arf2* mutations have an additive influence on the ABA-inhibited RAM activity, suggesting that ARF2 and PLT1 do not work in the same pathway. The reduced expression of *PLT1* in the *arf2-101* mutant might be caused by the indirect influence of ARF2. Interestingly, the expression of *PLT2* at both the transcriptional and translational level is slightly lower in the *arf2-101* mutant than in the WT under normal growth conditions. ABA treatment reduced the transcriptional level of *PLT2* to a similar level in the *arf2-101* mutant and in the wild type and consistently reduced PLT2 in the wild type, but did not change PLT2 protein in the *arf2-101* mutant. Given that *PLT2* transcripts are significantly reduced by ABA, the unchanged PLT2 protein level suggests that PLT2 is more stable in the *arf2-101* mutant than in the WT under ABA treatment. As ARF2 does not directly

regulate *PLT2* expression, it is likely that other factors regulated by ARF2 are involved in mediating the stability of the PLT2 protein. Using a DEX-inducible transgenic line, *35S:PLT2-GR*, we showed that after DEX induction, cell division was greatly promoted and cell differentiation was completely blocked in the *arf2-101* mutant, which did not occur in the WT. This result suggests that ARF2 antagonistically functions with PLT2 in modulating cell division and differentiation. However, ABA treatment could alleviate the positive role of PLT2 in cell division in the *arf2-101* mutant, suggesting that ABA signaling could also inhibit cell division independent of ARF2. Genetic analysis suggests that *ARF2* and *PLT2* likely work in the same pathway to regulate root growth. Although PLT2 protein accumulated to a higher level in the *arf2-101* mutant than in the WT, the *arf2-101* mutant still exhibits ABA hypersensitivity in RAM activity, suggesting that PLT1, PIN proteins, and other factors must be involved in the ARF2-mediated root growth under ABA signaling. This also suggests that when PLT2 exceeds a certain level, it will promote cell division and retard cell differentiation, which is mediated by ARF2.

In summary, our study uncovered important roles of ARF2 in coordinating with PLETHORAs and PINs to mediate root meristem activity under ABA signaling in *Arabidopsis*.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana plants of the *Columbia* ecotype (Col-0), *Enkheim-2* ecotype (En) and *Wassilewskija* ecotype (Ws) were used in this study. The marker lines and mutants used were: *ProPLT1-CFP*, *ProPLT2-CFP*, *ProPLT1:PLT1-YFP* and *ProPLT2:PLT2:YFP* (Galinha et al. 2007), *ProDR5-GFP* (Benkova et al. 2003), *ProPIN1:PIN1-GFP* (Benkova et al. 2003), *ProPIN4:PIN4-GFP* (Vieten et al. 2005), *ProPIN3:PIN3-GFP* and *ProPIN7:PIN7-GFP* (Blilou et al. 2005), *arf2-101* (Wang et al. 2011), *pin 1-1* (Okada et al. 1991), *pin4-3* (Friml et al. 2004), *pin7-2* (Benkova et al. 2003), *plt1-4*, *plt2-2* and *plt1-4plt2-2* (Aida et al. 2004), *Pro35S:PLT2-GR* (Ding and Friml, 2010) and *Pro35S:PIN1* (Benkova et al. 2003).

Seeds were surface-sterilized in 0.5% NaClO solution for 15 min, rinsed five times with sterile water, plated on Murashige and Skoog (MS) medium with 2.0% sucrose and 0.9% agar, and then stratified at 4 °C in the dark for 2 d. Plants were germinated and grown under long-day conditions (16 h light/8 h dark) at 20–22 °C with 60%–70% relative humidity.

For DEX induction analyses, 4-day-old uniform seedlings were transferred to MS medium or MS supplemented with 30 μM ABA, 2 μM DEX, or 30 μM ABA plus 2 μM DEX and vertically grown for 3 d before analysis. Alternatively, *Arabidopsis* seeds were germinated and grown on MS medium or MS medium containing 0.3 μM ABA, 2 μM DEX, or 0.3 μM ABA plus 2 μM DEX for 6 d before analyzing.

The primary root length (mm) and relative root growth (%) were measured from photographs of plates, using the Image J program (Image J; National Institutes of Health; http://rsb.info.nih.gov/ij). Twenty-five roots were measured from two Petri dishes for each treatment and three independent replicates were conducted for each condition.

Microscopy and confocal microscopy

Four- or 5-day-old uniform seedlings were transferred to MS medium supplemented with different concentrations of ABA, or DEX. After 3 or 5 d, the roots were photographed with an Olympus BX53 microscope. The meristem cell number was measured as the number of meristematic cortex cells from the quiescent center (QC) to the first elongated cell. The root meristem cell length was assessed as the distance between the QC and the first elongating cell (Perilli and Sabatini, 2010). The elongation zone (EZ) cell length and cell number was assessed as the distance from the first elongating cell to the first cell with a root hair.

For confocal microscopy images, 4-day-old uniform seedlings were transferred to MS medium or MS medium with 0, 10 or 30 μM ABA grown for 2 d. The seedlings were mounted in 10 μM propidium iodide for 3 min before being imaged with a Carl Zeiss LSM510 META confocal microscope. The relative fluorescence (%) was measured using Image J.

ACKNOWLEDGMENTS

This work was supported by grants from National Basic Research Program of China (973 program, 2012CB114300) and National Science Foundation of China (31421062) to Z. G.

AUTHOR CONTRIBUTIONS

Z. G. and S.P. designed the experiments. S. P. performed most of experiments and analyzed the data. Other authors assisted in experiments and discussed the results. Z. G. and S. P. wrote the manuscript.

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SUPPORTING INFORMATION

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Figure S1. The *pin1 arf2-101* double mutant is more resistant to ABA than the *arf2-101* single mutant in primary root growth

(A) The root growth phenotypes of the Col-0 and En ecotypes, and the *arf2-101*, *pin1-1*, (En) and *arf2-101 pin1-1* mutants. Five-day-old seedlings were transferred onto MS medium or MS medium containing 10 or 30 μM ABA and grown for 5 d. **(B)** Primary root length in **(A)**. **(C)** Relative root growth in **(B)**. Three experiments were conducted with similar results. Each experiment was conducted with 8-15 roots from three plates.

Figure S2. The *arf2-101 pin3-4* double mutant exhibits similar ABA sensitivity as the *arf2-101* single mutant in root growth

(A) Five-day-old seedlings were transferred onto MS medium or MS medium containing 10 or 30 μM ABA and grown for 5 d. **(B)** Primary root length in **(A)**. **(C)** Relative root growth in **(B)**. Three experiments were conducted with similar results. Each experiment was conducted with 8-15 roots. Values are means ± *SD*. Means with different letters are significantly different at $P < 0.01$. **(D)** Root meristem phenotypes of Col-0 (WT) and the *arf2-101*, *pin3-4*, and *arf2-101 pin3-4* mutant seedlings in **(A)**. The meristem zone is marked with a red line. Bars = 50 μm.

Figure S3. The *arf2-101 pin4-3* double mutant is more resistant to ABA than the *arf2-101* single mutant in primary root growth

(A) The root growth phenotypes of the Col-0 and En ecotypes, and the *arf2-101*, *pin4-3* (EN) and *arf2-101 pin4-3* mutants. Four-day-old seedlings were transferred onto MS medium or MS medium containing 10 or 30 μM ABA and grown for 5 d. **(B)** The primary root length of the seedlings in **(A)**. **(C)** The relative root length in **(B)**. Three experiments were conducted with similar results and each experiment was conducted with three replicates, each with 8-15 roots from one plate. Values are means ± *SD*. Means with different letters are significantly different at $P < 0.01$.

Figure S4. The *arf2-101 pin7-2* double mutant showed similar ABA sensitivity as the *arf2-101* single mutant in root growth

(A) Four-day-old seedlings were transferred to MS medium or MS medium containing 10 or 30 μM ABA and grown for 5 d. **(B)** The primary root length of the seedlings in **(A)**. **(C)** The relative root length in **(B)**. Three experiments were conducted with similar results. Each experiment was conducted with three replicates, each with 8-15 roots from one plate. Values are means ± *SD*. Means with different letters are significantly different at $P < 0.01$. **(D)**. Root meristem phenotypes of Col-0 (WT) and the *arf2-101*, *pin7-2*, and *arf2-101 pin7-2* mutant seedlings in (A). The meristem zone is marked with a red line. Bars = 50 μm.

Figure S5. Overexpression of *PLT2* promotes root meristem cell division and inhibits cell differentiation during seed germination in the *arf2-101* mutant

(A) Seedling growth phenotypes after ABA and DEX treatment. Seeds were germinated on MS medium or MS medium supplemented with 2 μM DEX, 0.3 μM ABA or 0.3 μM ABA plus 2 μM DEX for 6 d. **(B)** Statistical analyses of root meristem number in **(C)**. The experiment was conducted with three replicates, each with 12−25 roots from one plate. Values are means ± *SD*. **(C)** Comparison of the root meristems of the *Pro35S:PLT2-GR/Col-0* (left) and *Pro35S:PLT2-GR/arf2-101* (right) transgenic seedlings on MS medium supplemented with 2 μM DEX. Cell division, but no cell differentiation, was observed in the root meristem of the *Pro35S:PLT2-GR/arf2-101* transgenic plants with DEX induction. Scale bars = 50 μm.