Control of Root Meristem Size by DA1-RELATED PROTEIN2 in Arabidopsis

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The control of organ growth by coordinating cell proliferation and differentiation is a fundamental developmental process. In plants, postembryonic root growth is sustained by the root meristem. For maintenance of root meristem size, the rate of cell differentiation must equal the rate of cell division. Cytokinin and auxin interact to affect the cell proliferation and differentiation balance and thus control root meristem size. However, the genetic and molecular mechanisms that determine root meristem size still remain largely unknown. Here, we report that da1-related protein2 (dar2) mutants produce small root meristems due to decreased cell division and early cell differentiation in the root meristem of Arabidopsis (Arabidopsis thaliana). dar2 mutants also exhibit reduced stem cell niche activity in the root meristem. DAR2 encodes a Lin-11, Isl-1, and Mec-3 domain-containing protein and shows an expression peak in the border between the transition zone and the elongation zone. Genetic analyses show that DAR2 functions downstream of cytokinin and SHORT HYPOCOTYL2 to maintain normal auxin distribution by influencing auxin transport. Further results indicate that DAR2 acts through the PLETHORA pathway to influence root stem cell niche activity and therefore control root meristem size. Collectively, our findings identify the role of DAR2 in root meristem size control and provide a novel link between several key regulators influencing root meristem size.

Organ growth in multicellular organisms is determined by coordinating cell proliferation and cell differentiation. In plants, postembryonic root growth is sustained by the root meristem, a specialized proliferative tissue present at the growing root tip (Dinneny and Benfey, 2008). In the Arabidopsis (Arabidopsis thaliana) root meristem, the mitotically inactive quiescent center (QC) cells and the surrounding mitotically active stem cells form the stem cell niche, which provides the source of cells for all tissues in roots (van den Berg et al., 1995; Scheres, 2007; Dinneny and Benfey, 2008). Stem cells generate transit-amplifying cells, which undergo additional division in the proximal meristem and then differentiate in the meristem transition zone, prior to rapidly expanding in length in the elongation zone (Ubeda-Tomas and Bennett, 2010). Therefore, root meristem size is maintained by the balance between two competing processes: cell division versus cell differentiation.

Cytokinin and auxin play an important role in regulating the cell division and differentiation balance in roots (Dello Ioio et al., 2008). A recent study has revealed that cytokinin and auxin control root meristem size by influencing the expression of SHORT HYPOCOTYL2 (SHY2) in opposite ways (Dello Ioio et al., 2008). SHY2 encodes an auxin/indole-3-acetic acid (IAA) inducible protein3 (IAA3), a repressor of auxin signaling (Kim et al., 1998; Tian et al., 2003), which represses the auxin-inducible expression of PIN-FORMED (PIN) auxin transport facilitator genes (Dello Ioio et al., 2008). The PIN genes are essential for the formation of a proper auxin maximum and therefore regulate cell division in the root meristem (Blilou et al., 2005). Auxin in the proximal meristem mediates degradation of the SHY2 protein, promoting cell division (Dello Ioio et al., 2008). Cytokinin activates SHY2 transcription in the transition zone through a cytokinin-dependent transcription factor, ARABIDOPSIS RESPONSE REGULATOR1 (ARR1; Dello Ioio et al., 2008). This results in the down-regulation of auxin signaling and PIN expression, promoting cell differentiation. SHY2 and BREVIS RADIX have also been proposed to antagonistically affect cell differentiation through a complex cross-regulatory module converging on the PIN3 gene (Scacchi et al., 2010). In addition, disruption of UPBEAT1 activity alters the balance of reactive oxygen species between the proliferation zone and the elongation zone, resulting in a delay in the onset of cell
differentiation (Tsukagoshi et al., 2010). However, this pathway functions independently of auxin and cytokinin signaling (Tsukagoshi et al., 2010).

Several factors involved in the regulation of stem cell niche activity are known to affect root meristem size in Arabidopsis. SHORT ROOT (SHR) and SCARECROW (SCR), which encode members of the GRAS family of putative transcription factors, are required for stem cell maintenance, and their loss-of-function mutants exhibit reduced root meristem size (Di Laurenzio et al., 1996; Helariutta et al., 2000; Sabatini et al., 2003). The PLETHORA (PLT) genes, which encode AP2 class transcription factors, are required to maintain stem cell niche activity and determine the position of the stem cell niche (Aida et al., 2004; Galinha et al., 2007). High levels of PLT activity promote stem cell identity; lower levels increase mitotic activity of stem cell daughters; and further reduction in levels is required for cell differentiation (Galinha et al., 2007). The plt1 plt2 double mutant forms short roots with reduced root meristem size (Aida et al., 2004). The PIN genes regulate PLT expression in the distal root meristem (Blilou et al., 2005).

In turn, the PLT genes are required for PIN gene transcription at the distal root tip (Blilou et al., 2005). The PLT genes have been shown to have a gradient of expression, which is thought to be a graded read-out of auxin distribution in the root meristem (Galinha et al., 2007; Grieneisen et al., 2007). However, it is unknown whether PLT1/PLT2 can mediate the effect of cytokinin and SHY2 by responding to altered auxin distribution caused by cytokinin and SHY2 (Dello Ioio et al., 2008).

We have previously revealed that the DA1 protein with two ubiquitin-binding motifs (UIMs) and a single LIM (for Lin-11, Isl-1, and Mec-3) domain sets final seed and organ size by restricting cell proliferation in Arabidopsis (Li et al., 2008). Here, we report the role of DAR1-RELATED PROTEIN2 (DAR2) with a single LIM domain and a putative zinc-binding domain in root meristem size control. dar2 mutants display reduced meristem size owing to decreased cell division and early cell differentiation in the root meristem. dar2 mutants have been recently reported as lateral root development3 (lrd3) mutants (Ingram et al., 2011), but it is unknown how LRD3 controls root meristem size. In this study, our results indicate that DAR2 functions genetically downstream of cytokinin and SHY2 to maintain auxin distribution by influencing polar auxin transport. We further reveal that DAR2 acts upstream of PLT1/PLT2 to influence root stem cell niche activity and thus control root meristem size. Therefore, DAR2 provides a novel link between several key regulators influencing root meristem size.

RESULTs

dar2 Mutants Reduce the Number of Dividing Cells and Promote Cell Differentiation in the Primary Root Meristem

We previously revealed that DA1 acts redundantly with DAR1 to set final seed and organ size by restricting cell proliferation (Li et al., 2008). DA1 encodes a putative ubiquitin receptor with two UIMs and a single LIM domain defined by its conservation with the canonical Lin-11, Isl-1, and Mec-3 domains (Li et al., 2008). In contrast, DAR2 contains a single LIM domain and a putative zinc-binding domain but lacks UIM motifs compared with DA1 and DAR1 (Fig. 1A), suggesting a possible functional divergence between DA1 and DAR2. LIM domains, which mediate protein-protein interactions, are involved in a variety of cellular processes (Dawid et al., 1998; Kadmas and Beckerle, 2004). To characterize the role of DAR2 in plant growth and development, we obtained transferred DNA (T-DNA)-inserted loss-of-function mutants of the DAR2 gene. dar2-1 (SALK_016122) with the T-DNA insertion in the fourth intron of DAR2 is in the Columbia (Col-0) background, whereas dar2-2 (GT_5_109397) with the T-DNA insertion in the fifth intron is in the Landsberg erecta (Ler) background (Fig. 1A). The T-DNA insertion sites were confirmed by PCR using T-DNA-specific and flanking primers and sequencing PCR products (Supplemental Fig. S1, A and B). Quantitative reverse transcription (RT)-PCR analysis showed that dar2-1 produced approximately 14% of the wild-type level of DAR2 transcript in seedlings, while dar2-2 is a null allele (Supplemental Fig. S1C). Unexpectedly, dar2-1 and dar2-2 mutants produced shorter primary roots than their respective wild types (Fig. 1B). As early as 3 d after germination (DAG), the growth rate of primary roots was dramatically decreased in dar2 mutants (Fig. 1C). At 9 DAG, the primary root length of dar2 was about 57% of that of their respective wild-type controls (Fig. 1C). A plasmid containing a genomic copy of DAR2 (gDAR2) was introduced into the dar2-1 mutant. Nearly all transgenic lines exhibited complementation of dar2-1 root phenotypes (Fig. 1, D and J), indicating that DAR2 is required for root growth. We further investigated the root length of DAR2-overexpressing transgenic lines. However, root length was similar to that of wild-type roots (Supplemental Fig. S1, D–F), suggesting that DAR2 may act redundantly with other factors to promote root meristem growth.

As the root meristem size is correlated with root length (Ubeda-Tomáš et al., 2009), we investigated root meristems of the wild type and dar2 mutants. As shown in Figure 1, E to H, the size of dar2 root meristems was obviously smaller than that of wild-type root meristems. In agreement with the reduced size of the primary root meristem, development of epidermal root hairs started much closer to the initials in dar2-1 than in the wild type (Fig. 1O). The number of root meristem cells, which is defined by counting the number of cortical cells in a file extending from the initial cell adjacent to the QC to the first elongated cell (Dello Ioio et al., 2007), was dramatically reduced in dar2 mutants (Fig. 1I). To further substantiate the role of DAR2 in controlling root cell division, we marked cells in the G2/M stage of the cell cycle with the reporter CYCB1;1pro:CDB-GUS/GFP (Ubeda-Tomáš et al.,
The expression domain of CYCB1;1pro:GUS/GFP in dar2-1 was significantly decreased compared with that in the wild type (Fig. 1, K–N), indicating that dar2-1 reduces the population of dividing cells in the root meristem.

The reduced cell division could concurrently increase cell differentiation in the primary root meristem. Root meristem cells of dar2-1 were significantly longer than those of the wild type (Fig. 1P), suggesting that DAR2 influences cell elongation/differentiation in the root meristem.

**Auxin Affects DAR2 Expression**

The expression pattern of DAR2 was investigated in transgenic plants containing DAR2 promoter:GUS fusions (DAR2prom::GUS). During embryo development, no detectable GUS activity was observed (Supplemental Fig. S2, A–D). We further investigated the expression
pattern of DAR2 at postembryonic stages. At 1 DAG, GUS activity could be reliably detected in the vasculature of the primary root (Fig. 2A). At 5 DAG, DAR2 showed an expression peak in the border between the transition zone and the elongation zone (Fig. 2B; Supplemental Fig. S2H). The transition zone was defined according to previous reports (Verbelen et al., 2006; Baluska et al., 2010; Ivanov and Dubrovskyn, 2012). Cross sections through the transition zone of DAR2 pro:GUS primary roots revealed that DAR2 pro:GUS was expressed in pericycle and phloem but not in xylem (Fig. 2C). This expression pattern was further confirmed by using a genomic DAR2-GFP fusion construct (gDAR2-GFP; Supplemental Fig. S2, I and J), which can rescue dar2-1 phenotypes (Fig. 1, D and J).

Auxin regulates cell division, while cytokinin acts at the transition zone to control cell differentiation (Dello Ioio et al., 2008). Therefore, we asked whether DAR2 gene expression is influenced by auxin and cytokinin. Endogenous auxin generally promotes root growth, but supraoptimal auxin levels can inhibit root growth (Strader et al., 2011). Therefore, we treated DAR2 pro:GUS and gDAR2-GFP seedlings with 0.1 nM IAA and 5 μM IAA, respectively. These particular IAA concentrations were chosen because 0.1 nM IAA and 5 μM IAA have been shown to promote and repress root growth, respectively (Dello Ioio et al., 2008; Baskaran and Jayabalyn, 2009). As shown in Supplemental Figure S2, E and F, expression of DAR2 pro:GUS and gDAR2-GFP was not obviously affected by application of 0.1 nM IAA. By contrast, expression of DAR2 pro:GUS was reduced at 5 h and subsequent time points after application of 5 μM IAA (Fig. 2, D–F). Quantitative RT-PCR analysis showed that the expression of DAR2 was down-regulated by 0.1 nM IAA (Fig. 2I). Similarly, GFP fluorescence in gDAR2-GFP transgenic lines was also strongly decreased by exogenous application of 5 μM IAA (Fig. 2, G–1 and K). We next investigated the effect of cytokinin on DAR2 expression. As shown in Supplemental Figure S2G, the expression of gDAR2-GFP was not obviously altered by exogenous cytokinin application. Thus, these results indicate that 5 μM IAA represses DAR2 gene expression and protein levels.

**DAR2 Is Required for the Effects of Auxin and Cytokinin on Root Meristem Size**

Considering that the expression of DAR2 was down-regulated by exogenous application of 5 μM IAA (Fig. 2, D–K), we further asked whether DAR2 is involved in auxin-mediated control of root meristem size. In the wild type, exogenous application of 0.1 nM IAA resulted in an increase in the number of root meristem cells (Fig. 2L), consistent with previous results (Dello Ioio et al., 2008; Tsukagoshi et al., 2010). However, there was almost no effect on root meristem size of dar2-1 (Fig. 2L). In the wild type, treatment with 5 μM IAA led to a reduction in root meristem size because of a decrease in the number of root meristem cells (Fig. 2M). This reduction in cell numbers was less in dar2-1 root meristems (Fig. 2M). As auxin and cytokinin antagonistically regulate cell proliferation and cell differentiation in the root meristem (Dello Ioio et al., 2008), we asked whether the root meristems of dar2-1 had altered responses to cytokinin. In the wild type, treatment with 5 μM trans-zeatin (Zt) led to a decrease in the number of root meristem cells (Fig. 2N). By contrast, dar2-1 root meristems were less sensitive to cytokinin than wild-type root meristems. Collectively, these results show that DAR2 is required for the effects of auxin and cytokinin on root meristem size.

**DAR2 Acts Downstream of Cytokinin and SHY2 to Influence Root Meristem Size**

Auxin and cytokinin regulate root meristem size by controlling the abundance of SHY2 in opposite ways (Dello Ioio et al., 2008). Therefore, we asked whether DAR2 and SHY2 could function antagonistically in a common genetic pathway. To test this, we crossed dar2-2 with the shy2-2 gain-of-function mutant and generated a shy2-2 dar2-2 double mutant. The shy2-2 mutant formed short roots with smaller root meristems (Fig. 3, A and B), consistent with a previous study (Dello Ioio et al., 2008). The length of shy2-2 dar2-2 roots was similar to that of dar2-2 roots (Fig. 3A). The root meristem cell number of shy2-2 dar2-2 was indistinguishable from that of the dar2-2 single mutant (Fig. 3, B and H). We further crossed the shy2-31 loss-of-function mutant with dar2-2 and generated a shy2-31 dar2-2 double mutant. The number of cells in shy2-31 root meristems was significantly increased compared with that in wild-type root meristems (Fig. 3, B and H), consistent with a previous report (Dello Ioio et al., 2008). The root meristem cell number of shy2-31 dar2-2 was comparable with that of the dar2-2 single mutant (Fig. 3, B and H). Thus, the dar2-2 mutation is epistatic to shy2 with respect to root meristem size, indicating that the root meristem size phenotype of shy2 mutants is dependent on a functional DAR2.

To further understand the relationship between DAR2 and SHY2, we crossed shy2-2 and shy2-31 with the gDAR2-GFP line and generated gDAR2-GFP;shy2-2 and gDAR2-GFP;shy2-31 plants, respectively. Expression of gDAR2-GFP in shy2-2 or shy2-31 mutants was not altered compared with that in the wild type (Fig. 3, C, E, and G; Supplemental Fig. S3, A and C). We further treated gDAR2-GFP, gDAR2-GFP;shy2-2, and gDAR2-GFP;shy2-31 seedlings with 5 μM Zt. Expression of gDAR2-GFP in the wild type was not obviously affected by exogenous cytokinin application (Fig. 3, C, D, and G). By contrast, expression of gDAR2-GFP in shy2-2 was dramatically reduced in the elongation zone and the transition zone (Fig. 3, E–G), indicating that the shy2-2 gain-of-function mutation increases the effect of cytokinin on the expression of gDAR2-GFP. This result is consistent with the fact that root meristems of shy2-2 are hypersensitive to cytokinin (Dello
Auxin affects the expression of DAR2. A and B, DAR2pro:GUS expression in a 1-d-old seedling (A) and a 5-d-old primary root (B). The arrows indicate the border between the transition zone and the elongation zone. C, A cross section through the transition zone of the primary root shows that DAR2pro:GUS is expressed in pericycle and phloem. D to F, Auxin-regulated expression of DAR2pro:GUS. DAR2pro:GUS transgenic seedlings (4 DAG) were treated with 5 μM IAA for 0 h (D), 5 h (E), and 10 h (F) before GUS staining assays. G to I, Auxin-regulated expression of gDAR2-GFP. gDAR2-GFP transgenic seedlings (5 DAG) were treated with 5 μM IAA for 0 h (G), 5 h (H), and 10 h (I) before GFP assays. J, Analysis of auxin-regulated DAR2 expression in Col-0 and dar2-1 by quantitative real-time RT-PCR. Seedlings (4 DAG) were treated with or without 5 μM IAA for the indicated times. Values are given as means ± SD relative to the untreated wild type, set at 1.0. K, Fluorescence quantification of auxin-regulated gDAR2-GFP in Col-0 and dar2-1. Seedlings (4 DAG) were treated with or without 5 μM IAA for the indicated times. Values are given as means ± SD relative to the untreated wild type, set at 1.0. L to N, Average number of cortical cells in root meristems of Col-0 or dar2-1 seedlings after 24 h of treatment with auxin or cytokinin, respectively. The 4-d-old seedlings grown on MS medium were transferred to MS medium containing 0.1 nM IAA (L), 5 μM IAA (M), and 5 μM Zt (N). White arrowheads in D to I show the QC, and blue arrowheads indicate the cortex transition zone. Values in J to N are given as means ± SD. **P < 0.01 compared with their respective controls by Student’s t test. Bars = 100 μm (A, B, and D–I) and 20 μm (C). [See online article for color version of this figure.]

Figure 2. Auxin affects the expression of DAR2. A and B, DAR2pro:GUS expression in a 1-d-old seedling (A) and a 5-d-old primary root (B). The arrows indicate the border between the transition zone and the elongation zone. C, A cross section through the transition zone of the primary root shows that DAR2pro:GUS is expressed in pericycle and phloem. D to F, Auxin-regulated expression of DAR2pro:GUS. DAR2pro:GUS transgenic seedlings (4 DAG) were treated with 5 μM IAA for 0 h (D), 5 h (E), and 10 h (F) before GUS staining assays. G to I, Auxin-regulated expression of gDAR2-GFP. gDAR2-GFP transgenic seedlings (5 DAG) were treated with 5 μM IAA for 0 h (G), 5 h (H), and 10 h (I) before GFP assays. J, Analysis of auxin-regulated DAR2 expression in Col-0 and dar2-1 by quantitative real-time RT-PCR. Seedlings (4 DAG) were treated with or without 5 μM IAA for the indicated times. Values are given as means ± SD relative to the untreated wild type, set at 1.0. K, Fluorescence quantification of auxin-regulated gDAR2-GFP in Col-0 and dar2-1. Seedlings (4 DAG) were treated with or without 5 μM IAA for the indicated times. Values are given as means ± SD relative to the untreated wild type, set at 1.0. L to N, Average number of cortical cells in root meristems of Col-0 or dar2-1 seedlings after 24 h of treatment with auxin or cytokinin, respectively. The 4-d-old seedlings grown on MS medium were transferred to MS medium containing 0.1 nM IAA (L), 5 μM IAA (M), and 5 μM Zt (N). White arrowheads in D to I show the QC, and blue arrowheads indicate the cortex transition zone. Values in J to N are given as means ± SD. **P < 0.01 compared with their respective controls by Student’s t test. Bars = 100 μm (A, B, and D–I) and 20 μm (C). [See online article for color version of this figure.]
expression domain of DR5:GUS/GFP was obviously reduced in dar2-1 root meristems, although the expression maximum of DR5:GUS/GFP was still localized in the center of the root meristem (Fig. 4, A–D). Thus, this result shows that DAR2 affects normal auxin distribution in the root meristem. However, DAR2 was not expressed in the root tip, suggesting that DAR2 may influence auxin distribution in the root meristem in a non-cell-autonomous manner.

Auxin can be acropetally transported to the root meristem by auxin transporters (Dai et al., 2006). To determine whether the effect of dar2-1 on the auxin distribution could be related to polar auxin transport, we examined acropetal auxin transport in primary roots of Col-0 and dar2-1. A significant reduction in auxin movement was observed by measuring the transport of radiolabeled auxin in dar2-1 (Fig. 4Q), indicating that DAR2 is required for normal acropetal auxin transport. This result also shows that the reduced auxin transport contributes, at least in part, to the altered auxin distribution in dar2-1 root meristems.

Auxin efflux transporter PIN proteins are known to be critical for auxin distribution in the root meristem (Bilou et al., 2005). We monitored the expression of PIN1, PIN3, and PIN7 genes. These particular PIN genes were chosen because they are expressed in the vascular tissue transition zone and because a role of these genes in root meristem size control has already been established (Bilou et al., 2005; Dello Ioio et al., 2008). As shown in Supplemental Figure S5A, the mRNA expression level of PIN7 was reduced in dar2-1 compared with that in the wild type. By contrast, expression of PIN3 and PIN7 was increased in the DAR2-overexpressing line, although overexpression of DAR2 did not increase root meristem size (Supplemental Figs. S1, D–F, and SB). It can be suggested that the PIN genes may promote root meristem growth in a dose-dependent manner. We further examined the fluorescence of GFP fusions to the auxin efflux carriers PIN1, PIN3, and PIN7. The expression level of PIN1pro:PIN1-GFP in dar2-1 was similar to that in the wild type (Fig. 4, E, G, and R). The expression level of PIN3pro:PIN3-GFP in dar2-1 primary roots was very slightly reduced compared with that in the wild type (Fig. 4, I, K, and S). The expression level of PIN7, as shown by the PIN7pro:PIN7-GFP fluorescence, was obviously down-regulated by the dar2-1 mutation (Fig. 4, M, O, and T). Thus, this result suggests that the reduced expression of PIN3 and PIN7 may influence polar auxin transport in dar2-1 primary roots.

Cytokinin has been shown to repress the expression of PIN1, PIN3, and PIN7 (Fig. 4, E, F, I, J, M, and N; Dello Ioio et al., 2008). The root meristems of the pin1 pin3 pin7 triple mutant exhibited less sensitivity to exogenous cytokinin (Dello Ioio et al., 2008). Mutation in dar2-1 caused reduced expression of PIN3 and PIN7 (Fig. 4, S and T). Similarly, the root meristems of dar2-1 also showed insensitivity to exogenous cytokinin (Fig. 2N). Therefore, we asked whether DAR2 could influence the cytokinin regulation of PIN1, PIN3, and PIN7 expression. Expression of PIN3-GFP and PIN7-GFP was less sensitive to exogenous cytokinin treatment in dar2-1 than in the wild type (Fig. 4, I–P, S, and T). These results support that DAR2 is involved in cytokinin regulation of PIN3 and PIN7 expression.

**DAR2 Affects Root Stem Cell Niche Activity**

Local auxin levels, established by intercellular transport and biosynthesis, mediate the maintenance or differentiation of stem cells in the Arabidopsis root...
Figure 4. 

**Figure 4.** *dar2-1* influences auxin transport and distribution. A and B, Expression patterns of the DR5pro:GFP reporter in Col-0 (A) and *dar2-1* (B) at 6 DAG. C and D, Expression patterns of the DR5pro:GUS reporter in Col-0 (C) and *dar2-1* (D) at 6 DAG. E and G, Expression patterns of PIN1pro:PIN1-GFP in Col-0 (E) and *dar2-1* (G) primary roots at 5 DAG. F and H, Expression patterns of PIN1pro:PIN1-GFP in Col-0 (F) and *dar2-1* (H) primary roots after 7 h of treatment with 5 μM Zt at 5 DAG. I and K, Expression patterns of PIN3pro:PIN3-GFP in Col-0 (I) and *dar2-1* (K) primary roots at 5 DAG. J and L, Expression patterns of PIN3pro:PIN3-GFP in Col-0 (J) and *dar2-1* (L) primary roots after 7 h of treatment with 5 μM Zt at 5 DAG. M and O, Expression patterns of PIN3pro:PIN3-GFP in Col-0 (M) and *dar2-1* (O) primary roots at 5 DAG. N and P, Expression patterns of PIN7pro:PIN7-GFP in Col-0 (N) and *dar2-1* (P) primary roots after 7 h of treatment with 5 μM Zt at 5 DAG. Q, Acropetal auxin transport measured in roots of Col-0 and *dar2-1* seedlings at 7 DAG. Values are given as means ± se. R, Quantification of PIN1pro:PIN1-GFP fluorescence as shown in E to H. S, Quantification of PIN3pro:PIN3-GFP fluorescence as shown in I to L. T, Quantification of PIN7pro:PIN7-GFP fluorescence as shown in M to P. Values in R to T are given as means ± so relative to the untreated wild type (WT), set at 1.0. **P < 0.01 compared with the respective controls by Student’s t test. Bars = 100 μm (A–P). [See online article for color version of this figure.]

Figure 5. 

**Figure 5.** *dar2-1* affects stem cell niche activity. A and B, Expression pattern of WOX5pro:GFP in Col-0 (A) and *dar2-1* (B) at 4 DAG. C and D, Double staining of the QC25:GUS reporter (blue) and starch granules (brown) in Col-0 (C) and *dar2-1* (D) at 4 DAG. White arrows indicate the CSC layer. Bars = 50 μm (A, B, E, and F) and 20 μm (C, D, G, and H). [See online article for color version of this figure.]

(Ding and Friml, 2010). The stem cell niche activity has been shown to be crucial for root meristem size control (Aida et al., 2004; Zhou et al., 2010). Therefore, we asked whether DAR2 could affect stem cell niche activity by influencing auxin transport and distribution in the root meristem (Fig. 4). To test this, we examined the cellular organization of the QC and its surrounding stem cells. In Arabidopsis, the QC consists of four well-defined cells: two in the front and two in the back. A single layer of columella stem cells (CSCs) is present between the QC and the differentiated columella cells containing starch granules (Fig. 5C; Kornet and Scheres, 2009). In the wild type, WOX5pro:GFP was expressed specifically in the QC cells (Sarkar et al., 2007; Fig. 5A). The expression pattern of WOX5pro:GFP in *dar2-1* was similar to that observed in the wild type (Fig. 5B). QC25 was also expressed in the QC cells in wild-type roots (Kornet and Scheres, 2009; Fig. 5C), but its expression was more frequently detected in the layer of CSCs in *dar2-1* than in the wild type (17% in the wild type [n = 100], 53% in *dar2-1* [n = 100], 4 DAG; Fig. 5, C and D).

The QC cells are considered to be relatively mitotic inactive and divide only occasionally to self renew (Vranstraen et al., 2009). We further investigated whether the mitotic activity of QC cells is altered in *dar2-1*. To test this, we cultured 2-d-old seedlings for 24 h in the presence of 5-ethyl-2'-deoxyuridine (EdU), which has been used to mark cell division in the root meristem (Vranstraen et al., 2009; Zhou et al., 2010). In wild-type root meristems, most meristematic cells incorporated EdU, shown by red fluorescent nuclei (Fig. 5E), while the QC cells marked with WOX5pro:GFP...
GFP only occasionally contained EdU-stained nuclei (Vanstraelen et al., 2009; Zhou et al., 2010). By contrast, the QC cells in dar2-1 root meristems incorporated EdU more frequently than those in wild-type root meristems (nine of 12 in dar2-1 versus three of 12 in the wild type [n = 12]; Fig. 5, E and F), indicating that the QC cells of dar2-1 have higher mitotic activities than those of the wild type.

In the root meristem, stem cells are maintained in an undifferentiated state by QC cells (van den Berg et al., 1997). As QC25 was more frequently expressed in CSCs in dar2-1 than in the wild type, we examined whether the CSCs in dar2-1 roots had undergone premature differentiation. In the wild type, CSCs lacked starch granules and were distinct from the differentiated columella cells containing starch granules (Fig. 5C). By contrast, dar2-1 mutant roots showed starch granules in the cells corresponding to the CSC layer (Fig. 5, C and D), indicating that the dar2-1 mutation causes the disruption of CSCs in root meristems. Consistent with this, expression of the J2341 marker, which was specifically expressed in CSCs in the wild type (Fig. 5G; Zhou et al., 2010), was strongly suppressed in dar2-1 (Fig. 5, G and H), demonstrating that DAR2 is required for the maintenance of CSC activity. Taken together, these results show that DAR2 affects stem cell niche activity.

DAR2 Acts through the PLT1/PLT2 Pathway to Influence Stem Cell Niche Activity

Stem cell niche activity in the root meristem is maintained by two parallel transcription factor pathways: the SCR/SHR pathway and the PLT pathway (Di Laurenzio et al., 1996; Helariutta et al., 2000; Sabatini et al., 2003; Aida et al., 2004). Therefore, we asked whether DAR2 could act through the SCR/SHR pathway or the PLT pathway to control root meristem size by influencing stem cell niche activity. To test this, we generated scr-3 dar2-1 and shr-2 dar2-1 double mutants and determined their root meristem size. The scr-3 dar2-1 double mutant possessed smaller root meristems than either of the single mutants (Fig. 6, A–D and G). Similarly, the root meristem size of shr-2 dar2-1 was essentially additive compared with their parental lines (Fig. 6, A, B, and E–G). In addition, neither expression nor localization of SCR and SHR, marked by SCRpro:SCR-GFP and SHRpro:SHR-GFP fluorescence, was altered in dar2-1 compared with those in their respective wild types (Fig. 6, H–K). These results indicate that DAR2 acts independently of the SCR/SHR pathway to influence root meristem size.

We then tested whether DAR2 acts through the PLT pathway using a plt1-4 plt2-2 dar2-1 triple mutant. The root meristem size of the plt1-4 plt2-2 dar2-1 triple mutant was similar to that of the plt1-4 plt2-2 double mutant (Fig. 7, A–D and P), indicating that plt1-4 plt2-2 is epistatic to dar2-1. This result suggests that DAR2 and PLT1/PLT2 act in the same genetic pathway. To further understand the interaction between DAR2 and PLT1/PLT2, we crossed dar2-1 with PLT1pro:CFP (for cyan fluorescent protein) and PLT2pro:CFP lines and generated PLT1pro:CFP;dar2-1 and PLT2pro:CFP;dar2-1 plants. The expression levels of PLT1pro:CFP and PLT2pro:CFP in dar2-1 were similar to those in the wild type (Supplemental Fig. S6, A–D). Quantitative RT-PCR analysis also revealed that mRNA expression levels of PLT1 and PLT2 in dar2-1 were not obviously altered compared with those in the wild type (Fig. 8). We further asked whether DAR2 could affect the expression levels of PLT1/PLT2 proteins. Therefore, we crossed dar2-1 with PLT1pro:PLT1-YFP (for yellow fluorescent protein) and PLT2pro:PLT2-YFP lines and generated PLT1pro:PLT1-YFP;dar2-1 and PLT2pro:PLT2-YFP;dar2-1 plants. The expression level of PLT1, as shown by PLT1pro:PLT1-YFP fluorescence, was dramatically reduced in dar2-1 compared with that in the wild type (Fig. 7, E and F). Similarly, YFP fluorescence in PLT2pro:PLT2-YFP;dar2-1 plants appeared much weaker than that in PLT2pro:PLT2-YFP plants (Fig. 7, G and H).
levels of PLT1-YFP and PLT2-YFP were further visualized by immunoblotting with antibodies specific for GFP. Consistent with confocal imaging, protein levels of PLT1-YFP and PLT2-YFP were significantly reduced in the *dar2-1* mutant (Fig. 7, N and O). Thus, DAR2 is required for the maintenance of normal PLT1 and PLT2 protein levels.

Previous studies showed that dexamethasone (DEX) induction of 35Spro:PLT2-GR plants led to significant increases of root meristem cell number (Galinha et al., 2007). Therefore, we crossed a 35Spro:PLT2-GR line with *dar2-1* to generate 35Spro:PLT2-GR;*dar2-1* plants. An induction with DEX for 2 d did not influence wild-type and *dar2-1* seedling growth severely (Fig. 7I). In the wild type, the number of root meristem cells was significantly increased after induction with DEX (Fig. 7, I, K, and Q), consistent with previous reports (Barkoulas et al., 2007; Kornet and Scheres, 2009). In *dar2-1*, the number of root meristem cells was also substantially increased after an induction with DEX (Fig. 7, L, M, and Q). DEX induction also complemented the *dar2-1* defect in CSCs (Fig. 7R). Thus, these results show that DAR2 acts through the PLT pathway to influence the root stem cell niche activity, which is crucial for root meristem size control.

**DAR2 Mediates the Effect of Cytokinin on the Expression of PLT1/PLT2**

DAR2 acts genetically downstream of cytokinin but upstream of PLT1/PLT2 to control root meristem size, suggesting a link between cytokinin and PLT1/PLT2. A
previous study has shown that cytokinin represses the expression of PLT1/PLT2 (Dello Ioio et al., 2008), suggesting that PLT1/PLT2 may mediate the effect of cytokinin on root meristem size. We further investigated the expression of PLT1/PLT2 in cytokinin biosynthetic and signaling mutants. As shown in Figure 8, the mRNA expression levels of PLT1/PLT2 were significantly increased in ipt5-2, ipt7-1, ahk3-3, and arr1-4 mutants compared with those in the wild type, further supporting that cytokinin affects the expression of PLT1/PLT2. The PLT transcription factors have been proposed to act as a graded read-out of auxin distribution in the root meristem (Galinha et al., 2007). Thus, the PLT transcription factors may mediate the effect of cytokinin by responding to changes in auxin distribution caused by cytokinin.

Considering that DAR2 acts genetically downstream of cytokinin to affect auxin distribution in the root meristem, we further asked whether DAR2 could mediate the effect of cytokinin on the expression of PLT1/PLT2. Therefore, we monitored the expression of the PLT1/PLT2 genes in ipt5-2 dar2-1, ipt7-1 dar2-1, ahk3-3 dar2-1, and arr1-4 dar2-1 double mutants. As shown in Figure 8, the mRNA expression levels of PLT1/PLT2 were lower in these double mutants than those in ipt5-2, ipt7-1, ahk3-3, and arr1-4 single mutants, indicating that the increased expression of PLT1/PLT2 in ipt5-2, ipt7-1, ahk3-3, and arr1-4 mutants is dependent on a functional DAR2. Thus, this result indicates that DAR2 mediates the effect of cytokinin on expression of PLT1/PLT2.

**DISCUSSION**

Root meristem size is maintained by the balance between cell proliferation and cell differentiation. Auxin and cytokinin interact to control the cell division and differentiation balance via SHY2, which represses the auxin-inducible expression of PIN genes (Dello Ioio et al., 2008). The PLT1/PLT2 transcription factors have been proposed to act downstream of auxin to maintain stem cell niche activity in the root meristem (Bilou et al., 2005; Galinha et al., 2007). In this study, we show that DAR2 functions as a factor influencing root meristem size and acts genetically downstream of cytokinin and SHY2 but upstream of PLT1/PLT2 to affect root meristem growth.

**The Small Root Meristem Size Phenotype of dar2/lrd3 May Not Be Correlated with Defects in Phloem Development and Delivery**

The dar2 mutants produced short roots as a result of the reduced root meristem size (Fig. 1, E–H). dar2 mutants have been recently described as lrd3 mutants (Ingram et al., 2011), but nothing is known about how LRD3 controls root meristem size. In this study, we have demonstrated that DAR2 acts downstream of cytokinin and SHY2 but upstream of PLT1/PLT2 to control root meristem size. lrd3 plants showed abnormalities in phloem morphology at 5 d, causing a decrease in long-distance delivery of phloem to the primary root tip (Ingram et al., 2011). However, phloem loading and export from the shoot appeared to be normal (Ingram et al., 2011). There was a subsequent spontaneous recovery of normal phloem morphology at 11 d, which was correlated with increased phloem delivery (Ingram et al., 2011). If defects in phloem development and long-distance delivery affect dar2 root meristem size, one would expect that the small root meristem size phenotype of dar2 could be spontaneously recovered at 11 d. In fact, the root meristem size of dar2-1 at 11 d was similar to that observed at 5 d (Fig. 1I; Supplemental Fig. S7), indicating that the small root meristem size phenotype of dar2-1 was not spontaneously recovered at 11 d. In addition, exogenous application of auxin has been reported to rescue phloem development defects and phloem delivery of lrd3 (Ingram et al., 2011). However, exogenous application of auxin did not rescue the small root meristem phenotype of dar2-1 (Fig. 2L). By contrast, root meristems of dar2-1 were less sensitive to auxin (Fig. 2L). These results suggest that defects in phloem development...
and long-distance delivery are not responsible for the small root meristem size phenotype of \(d\ar2-1\).

**DAR2 Encodes a LIM Domain-Containing Protein**

The **DAR2** gene encodes a protein with a single LIM domain and a putative zinc-binding domain (Fig. 1A). The zinc-binding domain is too general to help assign a function of **DAR2**. A large number of diverse LIM proteins have been found in animals, which have essential physiological roles in various biological processes, including the regulation of cytoskeleton organization, axon guidance, cell proliferation, and cell motility (Dawid et al., 1998; Kadras and Beckerle, 2004). In Arabidopsis, two distinct LIM gene subfamilies have been reported. The first subfamily consists of six genes, which encode proteins with two LIM domains that share similarity with animal Cys-rich proteins (Papuga et al., 2010). These LIM domain-containing proteins bind actin filaments and influence actin cytoskeleton organization (Papuga et al., 2010). The genes in the second subfamily encode **DA1** and seven DAR proteins with a single LIM domain (Li et al., 2008). The members of the **DA1** family are involved in seed and organ size control, resistance signaling, and cold response (Li et al., 2008; Yang et al., 2010). The LIM domain has been shown to serve as an interface for protein-protein interaction (Dawid et al., 1998; Kadras and Beckerle, 2004), although the biochemical properties of the LIM domain are not fully understood. It seems likely that the LIM domain of **DAR2** may mediate interactions with its target proteins.

**LDR3/DAR2** was reported to be expressed in the phloem (Ingram et al., 2011). By contrast, cross sections of the transition zone revealed that **DAR2proGUS** was expressed in pericycle and phloem (Fig. 2C). This expression pattern was further confirmed by using **gDAR2-GFP**, which can complement the short-root phenotype of **d\ar2-1** (Fig. 1, D and J; Supplemental Fig. S2J). The promoter sequences of **DAR2proGUS** and **gDAR2-GFP** are longer than that of **LDR3proGUS** (Supplemental Table S1; Ingram et al., 2011), suggesting that this discrepancy in expression pattern might result from differences in their promoter lengths. This discrepancy also suggests that **DAR2** might possess distinct expression patterns in different root regions or at different developmental stages.

**DAR2 Acts Genetically Downstream of Cytokinin and **SHY2**

The root meristems of **d\ar2** mutants exhibited both decreased numbers of dividing cells and early cell differentiation (Fig. 1, C, I–N, and P). Auxin controls cell division, while cytokinin functions to promote meristematic cell differentiation (Dello Ioio et al., 2008). Our results indicate that root meristems of **d\ar2-1** were less sensitive to auxin and cytokinin (Fig. 2, L–N). **IAA** at 5 \(\mu\text{M}\) repressed **DAR2** gene expression and protein levels in the vascular tissue transition zone (Fig. 2, D–K). However, 0.1 \(\mu\text{M}\) **IAA** did not obviously promote the expression of **DAR2**, although this concentration of auxin could promote root meristem growth (Fig. 2L). It is possible that 0.1 \(\mu\text{M}\) **IAA** may increase the activity of **DAR2**, although its expression was not altered. Considering that endogenous auxin usually promotes cell proliferation in the root meristem, **DAR2** may act as a positive factor to mediate auxin-regulated cell division in the Arabidopsis root meristem.

It is known that auxin and cytokinin control root meristem size by influencing the expression of **SHY2** in opposite ways (Dello Ioio et al., 2008). Our genetic analyses demonstrate that **d\ar2-2** is epistatic to **shy2** mutants with respect to root meristem size (Fig. 3H). The expression of **gDAR2-GFP** in **shy2-2** roots was clearly decreased in the elongation zone and the transition zone after cytokinin treatment (Fig. 3, C–G). **SHY2** was expressed in the more distal protophloem and eventually throughout the vascular cylinder in the elongation zone (Scacchi et al., 2010). Thus, **DAR2** and **SHY2** have partially overlapping expression patterns in phloem cells (Scacchi et al., 2010). Cytokin-in biosynthesis and signaling mutants formed large root meristems by repressing the expression of **SHY2** (Dello Ioio et al., 2007). Genetic analyses show that the large root meristem phenotype of these cytokinin mutants is dependent on a functional **DAR2** (Fig. 3I). Thus, our findings reveal that **DAR2** acts genetically downstream of cytokinin and **SHY2** to influence root meristem size.

**DAR2 Influences Auxin Transport and Distribution**

Cytokinin controls root meristem activity by modulating the polar auxin transport (Dello Ioio et al., 2008; Ruzicka et al., 2009). The root meristems of the **pin1 pin3 pin7** triple mutant showed reduced sensitivity to cytokinin (Dello Ioio et al., 2008), suggesting that a proper auxin distribution in the root meristem is necessary for mediating the effect of cytokinin on root meristem size. The expression domain of **DR5proGUS** was reduced in the **dar2-1** mutant (Fig. 4, A–D), indicating that **DAR2** affects auxin distribution in the root meristem. Our data demonstrated that the **dar2-1** mutant reduced acropetal auxin transport in primary roots (Fig. 4Q). The reduced auxin transport can contribute, at least in part, to altered auxin distribution in **dar2-1**.

**PIN1**, **PIN3**, and **PIN7** have been shown to be expressed in the vascular tissue transition zone and to play an important role in controlling root meristem size (Bilou et al., 2005; Dello Ioio et al., 2008). Our results show that the expression levels of **PIN3proPIN3-GFP** and **PIN7proPIN7-GFP** in **dar2-1** were reduced in the transition zone (Fig. 4, I–P, S, and T). The decreased expression of **PIN3proPIN3-GFP** and **PIN7proPIN7-GFP** may contribute, in part, to the reduced acropetal auxin transport in **dar2-1**. We also observed that the expression of **PIN3proPIN3-GFP** and **PIN7proPIN7-GFP** was
clearly decreased in the root tip (Supplemental Fig. S8, A–D). As DAR2 was not expressed in the root tip, DAR2 might indirectly affect the expression of PIN3pro:PIN3-GFP and PIN7pro:PIN7-GFP in the root tip. It is possible that the altered auxin distribution in dar2-1 root meristems might cause feedback changes in the expression of PIN3 and PIN7 in the root tip. Cytokinin affects the expression of PIN1, PIN3, and PIN7 in the vascular tissue transition zone (Dello Ioio et al., 2008). Our data show that DAR2 is involved in the cytokinin regulation of PIN3 and PIN7 expression in the transition zone (Fig. 4, I–P, S, and T). However, cytokinin regulates PIN1 expression separately from DAR2 (Fig. 4, E–H and R).

DAR2 Acts in a Non-Cell-Autonomous Manner to Influence Root Stem Cell Niche Activity through the PLT Pathway

Auxin transport and biosynthesis-dependent auxin distribution affect the maintenance or differentiation of stem cells in the Arabidopsis root meristem (Ding and Friml, 2010). The dar2-1 mutation led to expanded domains of QC25 expression, increased mitotic activity of QC cells, and disruption of CSCs (Fig. 5), indicating that DAR2 is required for the maintenance of stem cell niche activity. Interestingly, DAR2 was strongly expressed in the border between the transition zone and the elongation zone, but not in root stem cells, suggesting that the DAR2 gene plays a non-cell-autonomous role in regulating root stem cell niche activity. As DAR2 affects auxin transport and local auxin distribution, auxin may mediate the effect of DAR2 on root stem cell niche activity.

It is generally believed that the PLT transcription factors act downstream of auxin to maintain stem cell niche activity in the root meristem (Bilou et al., 2005; Galinha et al., 2007). Our genetic analyses show that DAR2 and PLT1/PLT2 act in the same genetic pathway (Fig. 7P). Further results show that DAR2 is required for the expression of PLT1/PLT2, and overexpression of PLT2 can rescue, at least in part, the root stem cell niche activity of dar2-1 (Fig. 7, E–H and R), demonstrating that DAR2 functions genetically upstream of PLT1/PLT2 to influence stem cell niche activity. Thus, PLT2 may mediate the effect of DAR2 on root stem cell niche activity by responding to altered auxin distribution caused by DAR2. We also observed that DAR2 influences PLT1/PLT2 protein expression levels but not PLT1/PLT2 gene expression. DEX induction of 35Spro:PLT2-GR can complement the root stem cell niche phenotype of dar2-1. It can be explained that 35Spro:PLT2-GRdar2-1 plants with DEX induction might have enough functional PLT2 proteins to maintain stem cell niche activity due to higher expression of PLT2 (Supplemental Fig. S9), although the dar2-1 mutation decreased the expression of PLT2 proteins.

A Possible Link between Cytokinin and the PLT Pathway

The PLT transcription factors form a developmentally instructive protein gradient that is thought to be a read-out of an underlying auxin gradient in the root meristem (Galinha et al., 2007). However, it is unclear whether PLT1/PLT2 can mediate the effect of cytokinin by responding to changes in auxin distribution caused by cytokinin (Dello Ioio et al., 2008). A previous study showed that cytokinin represses the expression of PLT1/PLT2 (Dello Ioio et al., 2008), suggesting a possible link between cytokinin and the PLT pathway. A recent report indicated that alterations of PIN levels in the type-A ARR mutants result in changes in the distribution of auxin in root tips as well as an altered pattern of cell division and differentiation in the stem cell niche in the root apical meristem. This result suggests that cytokinin may indirectly control root stem cell niche activity by influencing auxin distribution in the root meristem.

We observed that expression of PLT1/PLT2 in ipt5-2, ipt7-1, ahk3-3, and arr1-4 mutants was significantly increased (Fig. 8). Further results show that up-regulation of PLT1/PLT2 in these cytokinin mutants requires DAR2 (Fig. 8), indicating that DAR2 mediates the effect of cytokinin on the expression of PLT1/PLT2. Cytokinin is synthesized in vascular tissues at the transition zone, suggesting that cytokinin indirectly affects the expression of PLT1/PLT2 by possibly influencing auxin transport and distribution in the root meristem. Interestingly, these cytokinin mutants did not exhibit obvious developmental changes in the root stem cell niche (Dello Ioio et al., 2007). It can be explained that the PLT1/PLT2 genes affect the root stem cell niche in a dose-dependent manner (Galinha et al., 2007). The up-regulation of PLT1/PLT2 in these cytokinin mutants may result in an increase in root stem cell niche activity (Galinha et al., 2007) and thus promote cell division in the root meristem.

Based on our results and previous data, we suggest a simple hypothesis for how DAR2 genetically interacts with cytokinin, auxin, SHY2, and PLT1/PLT2 to influence root meristem size in Arabidopsis. DAR2 acts genetically downstream of cytokinin and SHY2 to influence acropetal auxin transport and auxin distribution in the root meristem. At the same time, DAR2 also mediates the effect of auxin on root meristem size (Fig. 2, L and M). PLT1/PLT2 acts genetically downstream of DAR2 to mediate the effect of DAR2 on root stem cell niche activity by responding to altered auxin distribution caused by DAR2. Low levels of PLT1/PLT2 in dar2-1 cause reduced stem cell niche activity (Galinha et al., 2007), resulting in decreased cell division and early cell differentiation in the root meristem.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana) ecotypes Col-0, Ler, and Wassilewskija were used in this study. dar2-1 (SALK_016122) and dar2-2 (GT_5_109397) were obtained from the Arabidopsis Biological Resource Center. Some of the plant
Auxin Transport Assays

Auxin transport assays were conducted according to the method described previously with minor modifications (Peer et al., 2004). Briefly, 5-d-old seedlings were transferred to new MS agar plates. The root-shoot junctions were covered by the filter paper strips saturated in the solution (one-quarter-strength MS medium, 25 mM MES [pH 5.2], 500 nM unlabeled IAA, and 500 mM [3H]IAA). Seedlings were then placed upside down and incubated in the dark for 6 h. After incubation, apical 5-mm root sections were harvested and balanced in liquid scintillation solution (catalog no. 1200-439; Perkin-Elmer) for 24 h, and the assay was determined by liquid scintillation counting (Perkin-Elmer model 1450).

RNA Isolation and Quantitative Real-Time RT-PCR

Total RNA was extracted from apical 3-mm root sections of 5-d-old seedlings by using an RNeasy Plant Mini Kit (Qiagen). RT was performed as described previously (Li et al., 2006). Quantitative real-time RT-PCR analysis was performed with a Lightcycler 480 instrument (Roche) using the Lightcycler 480 SYBR Green 1 Master (Roche). ACTIN2 mRNA was used as an internal control. The specific primers for quantitative real-time RT-PCR are described in Supplemental Table S1.

Protein Extraction and Western-Blot Analysis

Apical 3-mm root sections from 5-d-old PLT1pro::PLT1-YFP, PLT2pro::PLT2-YFP, and PIN7pro::PIN7-YFP seeds were harvested, and the soluble proteins were extracted according to the method described previously with minor modifications (Li et al., 2002, 2012). Briefly, apical 3-mm root sections were ground to a fine powder in liquid nitrogen using a mortar and pestle. The powder was further ground in protein extraction solution (20 mM Tris-HCl [pH 8.8], 150 mM NaCl, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, and a portion of protease inhibitor cocktail [Roche]). The samples were spun at 140,000g for 10 min to deposit the insoluble cell debris (pellet). The supernatant was denatured by adding an equal volume of 2× sample buffer (125 mM Tris-HCl [pH 8.0], 5% SDS, 100 mM DL-dithiothreitol, and 0.04% bromophenol blue) and heating at 100°C for 10 min. The denatured proteins were resolved by SDS-PAGE and visualized by immunoblotting using an antibody to GFP (Beyotime). Cosmic Brilliant Blue-stained membranes are shown as loading controls.

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: ACTIN2 (AT1G18780), AKH3 (AT1G27520), AKRI (AT3G16857), CyxB1 (At4g37490), DAR2 (At1g53900), IPT5 (AT1G20400), IPT7 (At3g56630), PIN1 (At1g73590), PIN3 (At3g18840), PIN7 (At1g23280), PLT1 (At3g28940), PLT2 (At1g31190), SCR (At4g54220), SHR (At4g37650), SHY2 (AT1G02420), and WOX5 (At3g11260).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Molecular characterization of the DAR2 gene.

Supplemental Figure S2. Expression pattern of DAR2.

Supplemental Figure S3. DAR2 acts downstream of cytokinin.

Supplemental Figure S4. SHY2 does not physically interact with DAR2.

Supplemental Figure S5. Expression levels of PIN1, PIN3, and PIN7 in the wild type, dar2-1, and DAR2-OE-2.

Supplemental Figure S6. Expression patterns of PLT1::CFP and PLT2::CFP.

Supplemental Figure S7. Root meristem cell number of Col-0 and dar2-1 in MS medium with or without Suc at 11 DAG.

Supplemental Figure S8. Expression patterns of PIN3::PIN3-GFP and PIN7::PIN7-GFP.

Supplemental Figure S9. Expression levels of PLT2 in the indicated seedlings.

Supplemental Table S1. List of primers used in this study.

Supplemental Materials and Methods S1. Yeast two-hybrid assay.
ACKNOWLEDGMENTS

We thank Philip N. Benfey, Ben Scheres, Angus S. Murphy, Malcolm J. Bennett, Jason W. Reed, Tatsuo Kakimoto, Xiangdong Fu, Yunde Zhao, Chuanyou Li, Keke Yi, and Susana Ubeda-Tomas for kindly providing seeds used in this study; Chuanyou Li for the EdU incorporation assay kit; and the Arabidopsis Biological Resource Center and Nottingham Arabidopsis Stock Centre for dar2 mutants.

Received November 1, 2012; accepted January 3, 2013; published January 7, 2013.

LITERATURE CITED


DAR2 Controls Root Meristem Size