

Armadillo-related proteins promote lateral root development in *Arabidopsis*

Juliet C. Coates*[†], Laurent Laplace[‡], and Jim Haseloff[§]

*School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom; [‡]Unité Mixte de Recherche 1098 Institut de Recherche pour le Développement, BP 64501, 911 Avenue Agropolis, 34394 Montpellier cedex 5, France; and [§]Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, United Kingdom

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Armadillo/ β -catenin and related proteins have important functions during animal and *Dictyostelium* development, regulating cell differentiation, proliferation, and adhesion. Armadillo-repeat-containing proteins also exist in plants, but the majority have unknown roles. The *Arabidopsis* genes that show greatest sequence homology to Armadillo/ β -catenin are called ARABIDILLO-1 and -2. Here, we demonstrate that ARABIDILLO-1 and -2 promote lateral root development. *arabidillo-1/-2* mutants form fewer lateral roots, and ARABIDILLO-1-overexpressing lines produce more lateral roots than wild-type seedlings. ARABIDILLO–yellow fluorescent protein fusions are nuclear. ARABIDILLO proteins contain an F-box motif, and thus may target other proteins for proteasomal degradation. Overexpression of ARABIDILLO-1 protein fragments, including F-box fragments, in wild-type seedlings reduces lateral root formation to the level of the *arabidillo-1/-2* mutant. We have shown that plant β -catenin-related proteins regulate root development. We suggest that ARABIDILLO proteins may target an inhibitor of lateral root development for degradation and propose that *Arabidopsis* β -catenin-related proteins define a previously uncharacterized pathway that promotes root branching.

plant | β -catenin | arabidillo

Mammalian β -catenin and its *Drosophila* homologue Armadillo (Arm) are multifunctional proteins required for embryonic development and with roles throughout adult life (1, 2). These proteins specify cell fates by regulating gene expression and are regulated posttranscriptionally. Cytosolic β -catenin/Arm protein is targeted for destruction by the proteasome unless stabilized by extracellular Wnt signals. Wnt signals allow β -catenin to translocate to the nucleus and interact with LEF/TCF transcription factors. This protein complex can activate (or relieve the repression of) target genes (3, 4). β -catenin can also lead indirectly to transcriptional activation. For instance, the *Caenorhabditis elegans* β -catenin WRM-1 relocates the TCF-related repressor POP-1 from the nucleus to the cytosol (5). β -catenin/Arm is also required for the formation of adherens junctions, sites of cell–cell adhesion where transmembrane cadherin molecules are linked to the actin cytoskeleton by α - and β -catenin (1). This adhesive function of β -catenin is required for development (6–8).

In the multicellular protist *Dictyostelium*, a β -catenin-related protein, Aardvark, promotes prespore gene expression and is also found in actin-containing cell junctions (9). Thus, β -catenin functions are conserved outside the animal kingdom. However, the detailed mechanisms of Wnt/ β -catenin signaling have changed during evolution, with “noncanonical” signaling pathways defined in *C. elegans* and *Dictyostelium* (10–12).

β -catenin/Arm is part of a superfamily of metazoan Arm-repeat-containing proteins that regulate cell signaling, the cytoskeleton, and protein–protein interactions (13). A superfamily of Arm-repeat-containing proteins is also present in the plant kingdom (13, 14). Very little is known about the functions of the majority of these proteins. Plant Arm-repeat proteins have known roles in light/gibberellin signaling (15), self-incompatibility (15), trichome development (16, 17), abscisic acid signaling, (18) and receptor-kinase

signaling (19). However, apart from *Brassica* ARC1 (20), the mechanisms of protein function are largely unknown.

We wanted to discover the functions of ARABIDILLO-1 and -2, the two *Arabidopsis* proteins with greatest sequence homology to metazoan and *Dictyostelium* β -catenin (13). ARABIDILLO-1 and -2 are unique among *Arabidopsis* Arm-repeat proteins in having an F-box motif (13, 14) and fall into a phylogenetically distinct subgroup from other plant Arm-repeat proteins (14). Here, we show that ARABIDILLO-1 and -2 regulate root architecture by promoting root branching. Root architecture determines a plant’s ability to acquire water and nutrients and is regulated by many signals, including plant hormones (21) and nutrient status (22, 23), allowing the plant to adapt dynamically to a changing environment.

arabidillo-1/-2 mutants and ARABIDILLO-overexpressing seedlings can respond normally to exogenous lateral root-regulating signals such as auxin, suggesting that they regulate root branching by a previously undefined mechanism. We show that full-length ARABIDILLO-1 and -2 localize to the nucleus but that some parts of ARABIDILLO-1 can localize to the cytosol, suggesting that ARABIDILLO-1’s localization may depend on the availability of interaction partners, similar to β -catenin. Overexpression of ARABIDILLO protein fragments reduces lateral root formation, possibly by competing for endogenous ARABIDILLO-interacting proteins. We propose that ARABIDILLO-1 and -2 may target an inhibitor of lateral root development for proteasomal degradation.

Thus, we have shown that *Arabidopsis* proteins related to β -catenin/Arm define a previously uncharacterized pathway that promotes root branching, an important developmental process.

Results

Arabidopsis Genes Related to β -Catenin/Arm Are Expressed Throughout the Plant. As described previously, ARABIDILLO-1 and -2 show the greatest sequence homology of any *Arabidopsis* genes to *Dictyostelium* and metazoan β -catenin/Armadillo (13). There is a single ARABIDILLO homologue in the model monocot rice (accession no. AAP55033).

ARABIDILLO-1 and -2 contain nine Arm repeats (Fig. 1) and share 80% amino acid identity (92% within the Arm-repeat region). *Dictyostelium* β -catenin (Aardvark), ARABIDILLO-1 and -2, and *Oryza* ARABIDILLO have an F-box motif (residues 45–93 in ARABIDILLO-1, 38–85 in ARABIDILLO-2) (ref. 13 and Fig. 1). ARABIDILLO-1 is also annotated as *AtFBX5* (24). The F-box/Arm-repeat-domain structure is unique to these four proteins (www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi). ARABIDILLO-1 and -2 contain a basic nuclear-localization signal (NLS) at their N termini (Fig. 1; and see Fig. 6).

ARABIDILLO-1 and -2 mRNA is detected in all plant organs

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Abbreviations: Arm, Armadillo; GUS, β -glucuronidase; IAA, indole-3 acetic acid; MS, Murashige and Skoog medium; NLS, nuclear-localization signal; YFP yellow fluorescent protein; 2,4D, 2,4 dichlorophenoxyacetic acid.

[†]To whom correspondence should be addressed. E-mail: j.c.coates@bham.ac.uk.

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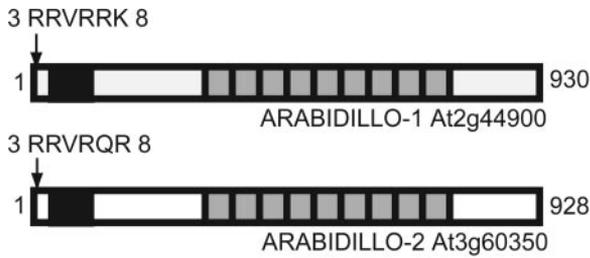


Fig. 1. ARABIDILLO-1 and -2 are *Arabidopsis* Arm-related proteins. Shown are ARABIDILLO-1 (At2g44900) and ARABIDILLO-2 (At3g60350) proteins. F-boxes (residues 45–93 in ARABIDILLO-1, 38–85 in ARABIDILLO-2), black; Arm repeats, light gray. Nuclear-localization sequences (residues 3–8) are marked with an arrow. Numbers indicate amino acids.

tested by RT-PCR (Fig. 2A). The spatial expression of *ARABIDILLO-1* and -2 was analyzed by fusing their putative promoters to the β -glucuronidase (*GUS*) gene. *pARABIDILLO-1::GUS* is active in both roots and shoots of seedlings (Fig. 2B). *GUS* activity is strongest in the root tip, pericycle, and vasculature (Fig. 2D). *pARABIDILLO-2::GUS* is also detected in both root and shoot (Fig. 2C), although strong expression is absent from mature primary and lateral root tips, where *GUS* activity is detected only in the columella (Fig. 2E). Both *ARABIDILLO* promoters are active in all cells of lateral root primordia (Fig. 2F and G), with mature lateral root expression patterns refining to resemble primary root patterns as the roots elongate and develop.

ARABIDILLO-1 and -2 Act Redundantly to Promote Lateral Root Development. To examine the *in planta* functions of *ARABIDILLO-1* and -2, *Arabidopsis* T-DNA insertion lines were obtained (ref. 25 and Fig. 3A). Neither the *arabidillo-1* nor -2 single mutant showed any obvious phenotype. *ARABIDILLO-1* and -2 are very similar to

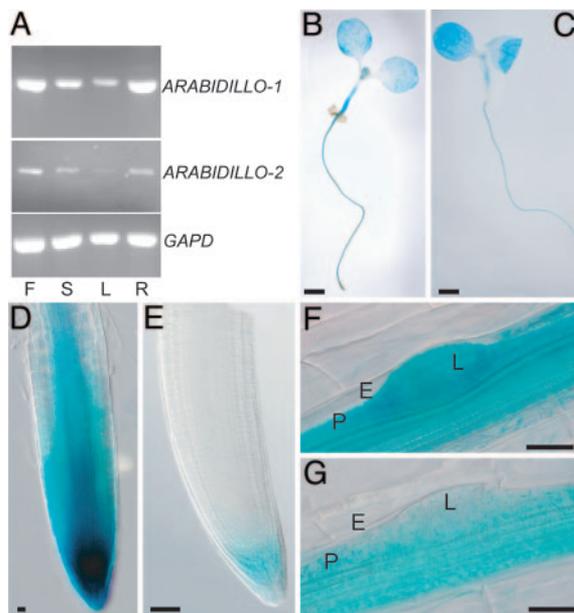


Fig. 2. ARABIDILLO genes are expressed throughout the plant. (A) RT-PCR of ARABIDILLO-1 and -2. F, S, L, and R, cDNA amplified from flowers, stems, leaves, and roots, respectively. GAPD, loading control. (B) *pARABIDILLO-1::GUS*. (C) *pARABIDILLO-2::GUS* 5-day seedling expression. (D) *pARABIDILLO-1::GUS*. (E) *pARABIDILLO-2::GUS* 5-day primary root-tip expression. (F) *pARABIDILLO-1::GUS*. (G) *pARABIDILLO-2::GUS* 8-day expression in lateral root primordia. E, endodermis; P, pericycle; LR, lateral root. [Scale bars, 1 mm (B and C), 25 μ m (D), 50 μ m (E), and 20 μ m (F and G).]

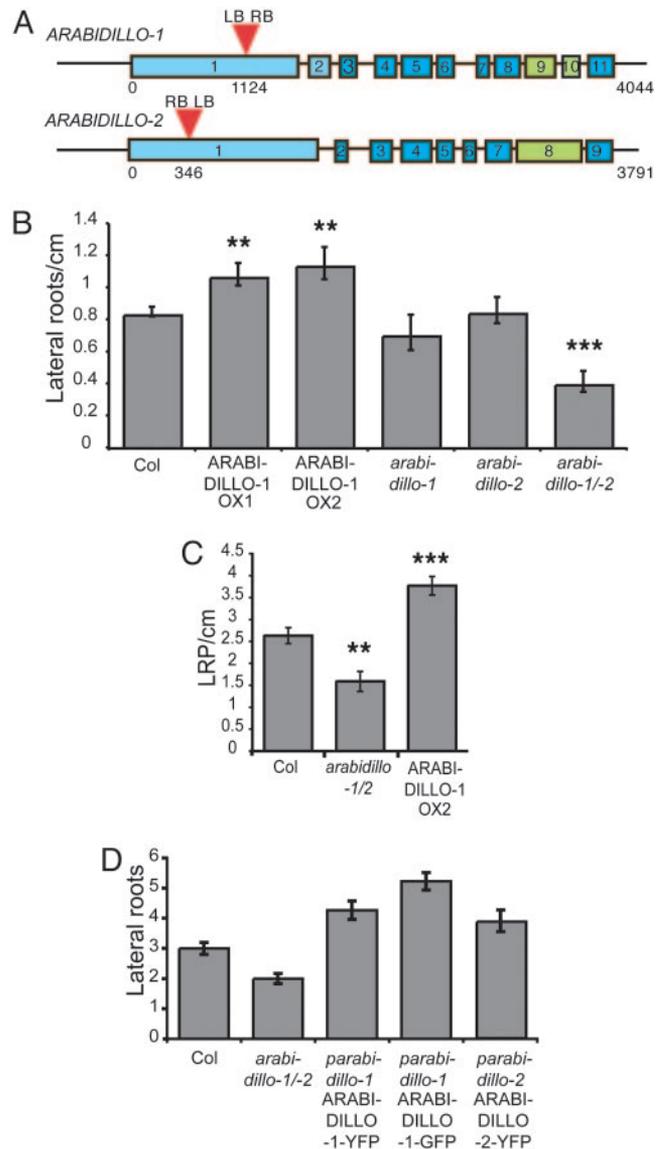


Fig. 3. *arabidillo-1* and -2 T-DNA insertion mutants. (A) T-DNA insertions (red) in ARABIDILLO-1 and -2. Blue/green boxes show corresponding exons (numbered) in each gene. Numbers below represent base pairs from the start codon. LB/RB, left/right T-DNA border. (B) Mean lateral root density in 11-day-old seedlings. Left to right, wild type; two independent 35S::ARABIDILLO-1-YFP lines (ARABIDILLO-1 OX1 and ARABIDILLO-1 OX2); *arabidillo-1* mutant; *arabidillo-2* mutant; *arabidillo-1/-2* mutant. Error bars \pm SEM. Mean root lengths: Col, 4.00 cm \pm 0.05 cm; ARABIDILLO-1 OX1 3.96 cm \pm 0.11 cm; ARABIDILLO-1 OX2 4.07 cm \pm 0.09 cm; *arabidillo-1* 4.21 cm \pm 0.11 cm; *arabidillo-2* 4.33 cm \pm 0.10 cm; and *arabidillo-1/2* 3.58 cm \pm 0.06 cm. *t* test, ***, $P < 0.001$ for *arabidillo-1/-2* and **, $P < 0.01$ for ARABIDILLO-1 OX1 and -2. (C) Mean lateral root primordia density at high magnification in 11-day-old seedlings. *t* test, **, $P < 0.01$ for *arabidillo-1/-2* and ***, $P < 0.001$ for ARABIDILLO-1 OX2. (D) Lateral roots are restored to *arabidillo-1/-2* by ARABIDILLO-fluorescent proteins. Left to right, mean lateral root number in wild type, *arabidillo-1/-2*, and independent *arabidillo-1/-2* lines expressing *pARABIDILLO-1::ARABIDILLO-1-YFP*, *pARABIDILLO-1::ARABIDILLO-1-GFP*, and *pARABIDILLO-2::ARABIDILLO-2-YFP*, respectively.

each other and show overlapping expression patterns and, thus, could be functionally redundant. Accordingly, when grown on vertical plates, *arabidillo-1/-2* double mutants develop fewer lateral roots than wild-type plants or either single mutant, despite having similar primary root lengths to wild type (Fig. 3B and C). The same result was obtained by using medium with 1% sucrose or basal

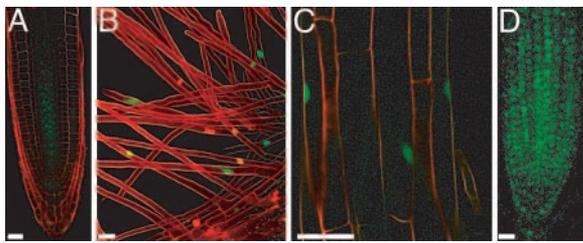


Fig. 5. ARABIDILLO-YFP fusion proteins are nuclear. (A) 35S::ARABIDILLO-1-YFP, root tip, confocal section. (B) 35S::ARABIDILLO-1-YFP, root hairs, confocal projection. (C) 35S::ARABIDILLO-2-YFP, root epidermal cells, confocal section. (D) 35S::ARABIDILLO-2-YFP, root tip confocal projection. Green, YFP; red, propidium iodide cell wall counterstain. (Scale bars, 25 μ m.)

(Fig. 6A) and expressed stably in *Arabidopsis*. For each protein, the subcellular localization of YFP was identified.

The first 93 aa (1–93) of ARABIDILLO-1 fused to YFP localize to the nucleus (Fig. 6B). Mutation of single residues in the NLS has no effect on the localization of residues 1–93, but mutation of pairs of residues (R4AR6A, R6AR7A, R6AK8A, or R7AK8A) moves localization away from the nucleus and into the cytosol (Fig. 6C–E). Deletion of the first 31 or 45 aa of this sequence [proteins 31–93 and 45–93 (the F-box), respectively] also leads to a partial relocation of the fusion protein to the cytosol (Fig. 6F and G). Addition of a GUS protein module to the F-box upstream of YFP (45–93:GUS, \approx 77 kDa) relocates the F-box to the cytosol (Fig. 6H), suggesting that the partial nuclear localization seen with proteins 1–93 (mutated), 31–93, and 45–93 is because their small size allows diffusion into the nucleus.

Expression of the entire N terminus of ARABIDILLO-1, up to the start of the Arm-repeat domain, (residues 1–376) or the N terminus without the NLS (residues 45–376) does not lead to YFP expression. This result might be due to a degradation signal in ARABIDILLO-1 between residues 93 and 221.

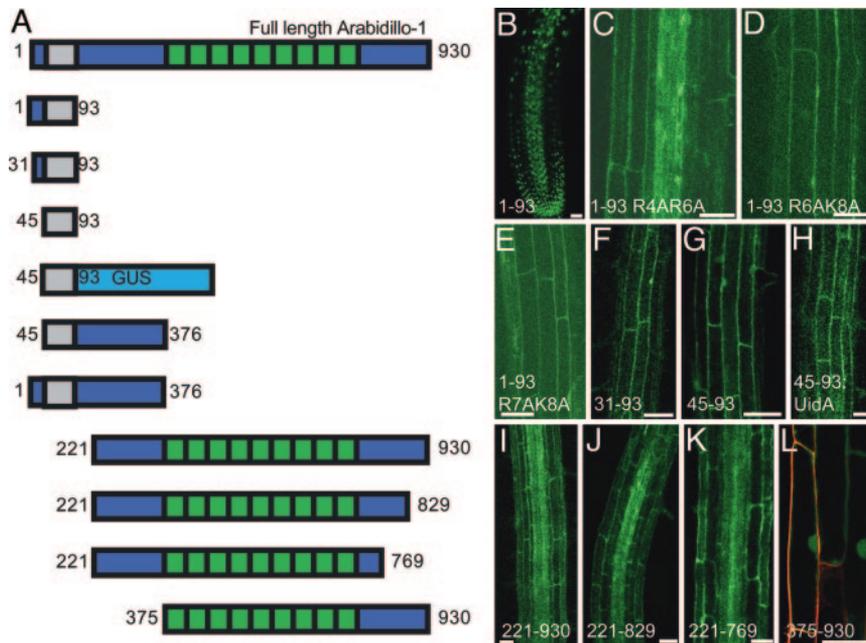


Fig. 6. ARABIDILLO-1 protein targeting. (A) Truncated YFP fusion proteins. Green boxes, Arm repeats; gray box, F-box (residues 45–93); dark blue, remainder of ARABIDILLO-1 protein; light blue, GUS. (B) Nuclear localization of residues 1–93. (C–E) Loss of nuclear localization in mutants (residues 1–93) R4AR6A, R6AK8A, and R7AK8A. (F and G) Cytosolic localization of residues 31–93 and 45–93. (H) Cytosolic localization of 45–93-GUS. (I–K) Cytosolic localization of residues 221–930 (I), 221–829 (J), and 221–769 (K). (L) Cytosolic and nuclear localization of residues 375–930. Representative root confocal sections are shown. Green, YFP; red, propidium iodide. (Scale bars, 25 μ m.)

A protein consisting of partial N terminus, the Arm-repeat domain and C-terminal region (residues 221–930) is cytosolic, as are similar constructs with truncated C termini (residues 221–769 and 221–829; Fig. 6 I–K). A protein containing only the Arm domain and C terminus (residues 375–930, >80 kDa) localizes to the nucleus and cytosol (Fig. 6L). The partial nuclear localization of residues 375–930 shows that ARABIDILLO-1 protein can localize to the nucleus in the absence of a classical NLS, presumably through its Arm-repeat domain, as is seen with β -catenin (34). There are two putative nuclear export signals (consensus L/VXXL/VXL/V) between residues 221 and 375, but the localization of residues 221–930 is not sensitive to Leptomycin B (J.C.C., unpublished work).

Overexpression of ARABIDILLO-1-YFP Protein Fragments Reduces Lateral Root Development. To understand how ARABIDILLO proteins promote lateral root development, we observed the effect of truncated ARABIDILLO-1-YFP protein overexpression on lateral root number. Wild-type seedlings expressing F-box fragments (1–93 and 45–93) form a similar number of lateral roots to *arabidillo-1/-2* mutants (Fig. 7), suggesting that F-box fragments block endogenous ARABIDILLO activity by disrupting the interaction between the full-length proteins and a binding partner(s), for example, component(s) of the proteasome.

Seedlings overexpressing ARABIDILLO-1 (residues 375–930)-YFP form a reduced number of lateral roots (Fig. 7), suggesting that this protein also inhibits endogenous ARABIDILLO function. None of the seedlings expressing ARABIDILLO protein fragments show additional phenotypes, suggesting that the fragments act as ARABIDILLO-specific “dominant negatives.”

Thus, we conclude that both the F-box and the C-terminal (Arm-repeat) domain are required for *in vivo* ARABIDILLO protein function and that both regions of the protein may interact with specific protein partners.

Discussion

β -Catenin-Related Proteins Promote Lateral Root Development in *Arabidopsis*. *Arabidopsis* possesses >100 Arm-repeat-containing proteins (14), of which ARABIDILLO-1 and -2 are most similar to

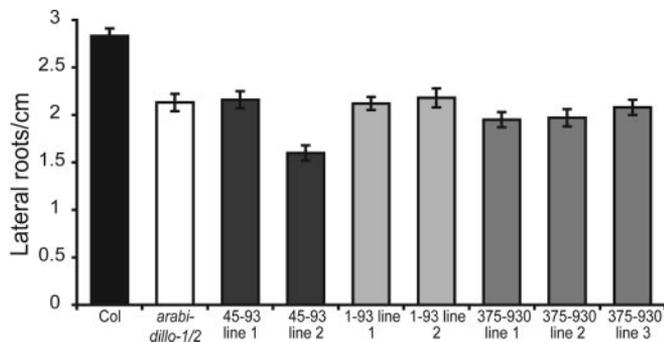


Fig. 7. ARABIDILLO-1-YFP protein fragments reduce lateral root density. Lateral root density of 11-day-old seedlings. Left to right, Wild type (Col, black); *arabidillo-1/-2* (white); wild-type seedlings expressing ARABIDILLO-1 (45–93) -YFP, 2 independent lines (dark gray); wild-type seedlings expressing ARABIDILLO-1 (1–93) -YFP, 2 lines (light gray); wild-type seedlings expressing ARABIDILLO-1 (375–930) -YFP, 3 lines (medium-gray). *t* test, $P < 0.001$ for all lines compared with wild type.

metazoan and *Dictyostelium* β -catenins (13). Here, we show that ARABIDILLO-1 and -2 promote lateral root development. *arabidillo-1/-2* mutant seedlings form fewer lateral roots than wild type and *arabidillo* single mutants, whereas plants overexpressing ARABIDILLO-1 have an increased lateral root number.

ARABIDILLO-1 and -2 Interact with Other Proteins to Promote Lateral Root Development. Overexpression of ARABIDILLO-1 protein fragments reduces lateral root formation, suggesting that the protein fragments act as dominant negatives, perturbing the function of endogenous ARABIDILLO proteins. Given their nuclear localization, ARABIDILLO-1 and -2 are likely to function by regulating gene expression either directly (as seen with nuclear β -catenin during “canonical” Wnt signaling) or indirectly. Overexpression of the C-terminal fragment (residues 375–930) of ARABIDILLO-1 could block lateral root formation by blocking a signaling function of the Arm domain, for example by forming a nonfunctional transcriptional complex on a target promoter, if N-terminal sequences are also required for transcriptional activation. However, N-terminally truncated animal β -catenin is able to activate transcription in certain contexts.

ARABIDILLO-1 and -2 contain an F-box motif. F-box proteins are specificity factors for multisubunit E3 ubiquitin ligases that degrade target proteins (35). Thus, ARABIDILLO-1 and -2 could target a protein(s), for example, an inhibitor of lateral root development, for proteasomal degradation. The Arm domain may, therefore, inhibit ARABIDILLO function by blocking the interaction of a lateral root inhibitor with the Arm domain of endogenous ARABIDILLO proteins, whereas F-box fragments would inhibit the degradation of target inhibitor(s) by blocking the interaction of endogenous ARABIDILLO with the proteasome.

ARABIDILLO-1 and -2 Do Not Function Within Known Lateral Root Signaling Networks. *arabidillo-1/-2* mutants formed fewer lateral roots than wild type, whereas ARABIDILLO-1-overexpressing plants formed more, under all conditions tested. The fact that neither mutant nor overexpressing plants were hypersensitive or resistant to hormonal or nutritional signals suggests that ARABIDILLO-1 and -2 can be added to the small number of known “intrinsic” lateral root regulatory genes (23).

ARABIDILLO-1 and -2 may regulate a competence or likelihood of pericycle cells to divide to form lateral roots. It is known that β -catenin stimulates cell proliferation through transcription of G1 cyclins (36–38). Overexpression of Kip-Related Protein-2, a G1-S-specific cyclin-dependent kinase inhibitor, reduces, but does not abolish, lateral root formation in *Arabidopsis* (39), similar to the

arabidillo-1/-2 mutant and, thus, could be a candidate for a lateral root inhibitor that ARABIDILLO-1 and -2 degrade independently of auxin signaling.

The *arabidillo* mutant and overexpressing phenotypes also resemble the root phenotypes of *Arabidopsis* heterotrimeric G protein-subunit mutants (40). Mutants in the β -subunit, *agb-1*, have widespread developmental effects, including a doubling of lateral root number. Conversely, *gpa-1* α -subunit mutants have half the lateral roots of wild type. Neither *GPA-1* nor *AGB-1* interact directly with auxin signaling. *GPA-1* and *AGB-1* may integrate multiple signals controlling cell proliferation (40, 41).

ARABIDILLO-1 Protein Targeting. We have shown that different regions of ARABIDILLO-1 can target YFP to different parts of the cell. The ARABIDILLO-1 N terminus contains a NLS. β -catenin does not contain a classical NLS, but the Arm-repeat domain allows nuclear targeting in an NLS-independent manner (34). The same is true of the Arm domain of ARABIDILLO-1. Whether the ARABIDILLO-1 NLS is regulated *in vivo* is not known.

Are ARABIDILLO-1 and -2 True Homologues of Animal and Dictyostelium β -Catenin? The phenotype of the *arabidillo-1/-2* mutant is subtle compared with the lethal effects of Arm/ β -catenin loss of function in animals, which leads to cell patterning defects and developmental arrest (42–46). *C. elegans* has three β -catenin homologues, *wrm-1*, *hmp-2*, and *bar-1*. Loss of *wrm-1* function is embryo-lethal because of a failure to specify endoderm (47). Loss of *hmp-2* arrests embryo development during gastrulation because of failed cell–cell adhesion (6). However, loss of *bar-1* is not lethal but causes postembryonic cell-fate defects in the vulva (48) and certain neurons (49).

Dictyostelium and plants reprogram their development in response to a changing environment. The *Dictyostelium* β -catenin (*aardvark*) mutant is not lethal. Although *Aardvark* functions analogously to β -catenin (9, 50), the *aardvark* mutant is able to complete multicellular development, as is *arabidillo-1/-2*.

Our data suggest that the mechanism by which ARABIDILLO-1 and -2 promote lateral root development may be distinct from the direct transcriptional function of animal β -catenin during canonical Wnt signaling. However, indirect transcriptional functions for β -catenin during Wnt signaling also exist; for example, *C. elegans* WRM-1 promotes the nuclear export of the LEF/TCF transcription factor POP-1 (5). *Dictyostelium Aardvark*, like ARABIDILLO-1 and -2, contains an F-box. The mechanism by which *Aardvark* regulates transcription and cell fate is unknown; *Aardvark* protein localizes to the cytosol in growing cells (9), suggesting that it may affect transcription indirectly.

Whether ARABIDILLO-1 and -2 resemble metazoan β -catenin by directly regulating transcription and/or cytoskeletal processes or whether they were coopted in plants for a distinct developmental mechanism remains to be established.

Methods

Cloning and Construct Generation. ARABIDILLO promoter sequences were amplified from Col-0 genomic DNA and inserted into pBI101 to make *pARABIDILLO::GUS* reporters. 35S::ARABIDILLO-YFP fusions were made in pGreen0029 (51). Full-length and truncated ARABIDILLO-1 and -2 coding regions were amplified from Col-0 genomic DNA. The ARABIDILLO-1 NLS was mutagenized by using QuikChange XL (Stratagene). Constructs in *Agrobacterium tumefaciens* GV3101 were transformed into *Arabidopsis* by floral dip (52).

Plant Growth Conditions. *Arabidopsis* seedlings were grown in sterile long-day conditions on 0.5 \times Murashige and Skoog (MS) medium (Sigma M0404), 1% agar, pH 5.7. Mature plants were grown in Levington M3 compost/vermiculite in the greenhouse.

RT-PCR. Total RNA was prepared from whole seedlings, mature aerial tissues, and seedling root tissue (Qiagen RNeasy plant miniprep). Gene-specific cDNA products were amplified from 50 ng of RNA by One-Step RT-PCR (Qiagen) with 32 cycles of PCR. The *GAPD* gene was amplified as a control.

GUS Staining and Microscopy. Seedlings were assayed for GUS activity according to standard protocols (53). Tissue was cleared through an ethanol series and mounted in Hoyer's medium. For live tissue imaging, Leica TCS and BioRad Radiance 2400 confocal microscopes were used. Seedlings were incubated in 5 mg/liter propidium iodide for 1.5 min and rinsed in distilled water before imaging.

Mutant Analysis. Homozygous, single-insert *arabidillo-1* and *-2* lines were identified from the Syngenta SAIL collection (25) and screened by segregation analysis, PCR with the recommended primers (25), and Southern blotting (DIG system; Roche). The Nucleon Phytopure kit (Amersham Pharmacia Biotech) was used for genomic DNA extraction. *arabidillo-1* and *-2* lines were crossed to generate double mutants.

Root Assays. Seedlings were grown vertically as a single row of seeds planted 1.5 cm from the top of 0.5× MS plates. Visible

lateral roots were counted 7–13 days after germination. Root length was measured from digital photographs by using the program IMAGEJ (<http://rsb.info.nih.gov/ij>). Seedling lateral root density was defined as lateral roots per cm of primary root.

For growth regulator experiments, 0.5× MS plates were supplemented with 1 μM IAA, 20 nM 2,4D, 0.5–1 μM abscisic acid, or the relevant solvent control.

Nutrient experiments were based on several protocols (54–56). Control plates (pH 5.7) were 20 mM NH₄NO₃, 3 mM CaCl₂, 1.5 mM MgSO₄, 19 mM KNO₃, 1.25 mM KH₂PO₄, 1× micronutrients (Bio-101 systems 5100–414), 0.5% sucrose, and 1% agar. Low-nitrogen plates contained 19 mM KCl instead of KNO₃ and 10 μM NH₄NO₃; MgSO₄ was substituted with MgCl₂ (low sulfur). Low phosphorous macronutrients were 2 mM NH₄NO₃, 0.3 mM CaCl₂, 1.9 mM KNO₃, and 1.25 mM KCl.

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