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AtMYB93 is a novel negative regulator of lateral root development in Arabidopsis

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Summary
• Plant root system plasticity is critical for survival in changing environmental conditions. One important aspect of root architecture is lateral root development, a complex process regulated by hormone, environmental and protein signalling pathways.
• Here we show, using molecular genetic approaches, that the MYB transcription factor AtMYB93 is a novel negative regulator of lateral root development in Arabidopsis.
• We identify AtMYB93 as an interaction partner of the lateral-root-promoting ARABIDILLO proteins. Atmyb93 mutants have faster lateral root developmental progression and enhanced lateral root densities, while AtMYB93-overexpressing lines display the opposite phenotype. AtMYB93 is expressed strongly, specifically and transiently in the endodermal cells overlying early lateral root primordia and is additionally induced by auxin in the basal meristem of the primary root. Furthermore, Atmyb93 mutant lateral root development is insensitive to auxin, indicating that AtMYB93 is required for normal auxin responses during lateral root development.
• We propose that AtMYB93 is part of a novel auxin-induced negative feedback loop stimulated in a select few endodermal cells early during lateral root development, ensuring that lateral roots only develop when absolutely required. Putative AtMYB93 homologues are detected throughout flowering plants and represent promising targets for manipulating root systems in diverse crop species.

Introduction
Plant rooting systems are fundamental for absorbing nutrients and water, anchoring the plant to its substrate, and responding to internal and external signals. As plants are sessile, plasticity of their root system is critical for survival. Plant root architecture requires complex regulation during development in response to hormones, signalling molecules and environmental changes.

The root systems of most vascular plants are formed by branching of lateral roots (LRs) from a primary root (PR) that first develops during embryogenesis. This process has been studied in great detail in several flowering plants, particularly Arabidopsis (Osmont et al., 2007; Nibau et al., 2008; De Smet, 2012). LRs initiate from a specialized cell layer in the PR, the pericycle. In Arabidopsis and most other dicots, LRs initiate from a specialized cell layer in the PR, the pericycle. In Arabidopsis and most other dicots, LRs are formed only from pericycle cells overlying the developing xylem tissue (the xylem pole pericycle). LR development involves stimulation and dedifferentiation of pericycle founder cells, which increase in size, re-enter the cell cycle, and divide asymmetrically to give rise to a lateral root primordium (LRP), which then emerges through the outer layers of the PR (Celenza et al., 1995; Laskowski et al., 1995; Malamy & Benfey, 1997; Casimiro et al., 2003; Kurup et al., 2005; Peret et al., 2009a; Vermeer et al., 2014). The endodermis, the cell layer immediately overlying the pericycle, has recently been identified as a key regulator of LR developmental progression (Duan et al., 2013; Marhavy et al., 2013; Vermeer et al., 2014). Feedback from the endodermis to the pericycle is required for LR initiation (Marhavy et al., 2013; Vermeer et al., 2014). Moreover, the endodermis undergoes local remodelling and morphological changes during the very early stages of LR development, to accommodate the developing LRP (Vermeer et al., 2014), and also regulates later LR emergence events (Duan et al., 2013).

LR development and changes in root architecture are brought about through a combination of hormone signalling, environmental cues and hormone-independent protein activity (Osmont et al., 2007; Nibau et al., 2008; Tian et al., 2014). The key hormone in the development of LRs is auxin, which regulates all...
stages of LR development (Osmont et al., 2007; Nibau et al., 2008; Fukaki & Tasaka, 2009; Peret et al., 2009a). LR development is also affected by the majority of other plant hormones (Osmont et al., 2007; Xue & Zhang, 2007; Nibau et al., 2008; Sun et al., 2009; Kapulnik et al., 2011; Ruyter-Spira et al., 2011; Duan et al., 2013), and crosstalk between hormones occurs (Fukaki & Tasaka, 2009).

A diverse range of proteins and transcription factors integrate the signals controlling LR development, as do proteins that control LR development in an apparently hormone- and signal-independent manner, referred to as ‘intrinsic’ LR regulators (Malamy, 2005; Osmont et al., 2007; Hruz et al., 2008; Nibau et al., 2008). ARABIDILLO proteins are one example of putative intrinsic LR regulators: ARABIDILLO1 and ARABIDILLO2 act redundantly to promote LR development (Coates et al., 2006; Nibau et al., 2011).

In this paper, we show that a small, previously uncharacterized subfamily of R2R3 MYB (myeloblastosis) transcription factors interacts with ARABIDILLO1, and that at least two of the MYBs play a role during LR development. We show that one member of this subfamily, AtMYB93, is expressed exclusively and transiently in roots in the endodermal cells overlying developing LRPs. Mutant and overexpression analyses demonstrate that AtMYB93 functions as a negative regulator of LR development. Furthermore, we show that AtMYB93 is induced by auxin, and that Atmyb93 mutants are insensitive to auxin specifically with respect to LR development. We propose that AtMYB93 is part of a novel negative feedback loop stimulated specifically in the endodermis upon LR initiation to ensure that LRs are formed only in the correct place.

Materials and Methods

PCR primers

All primers used are listed in Supporting Information Table S1.

Yeast two-hybrid screening/assays

The ARABIDILLO1 ARMADILLO (ARM) domain (amino acids 378–767) was cloned into pGBK T7 and used to screen a seedling root primary cDNA library (Sorrell et al., 2003). AtMYB93, AtMYB53, AtMYB75/PAP1 (PAP1 = PRODUCTION OF ANTHOCYANIN PIGMENT 1) and AtMYB91/AS1 (AS1 = ASYMMETRIC LEAVES1) cDNAs were PCR-amplified from whole-seedling total RNA and cloned into pGADT7. Constructs were co-transformed into yeast strain AH109 and tested for protein–protein interactions following the manufacturer’s protocols (Takara Biosciences, Otsu, Japan). We could not test the reciprocal interaction, as AtMYB92 autoactivates the yeast two-hybrid system.

Sequence analysis, alignment and phylogeny

The initial MYB alignment (Fig. 1c) was conducted using CLUSTALX (Larkin et al., 2007) using the default settings. Alignments were annotated using BOX SHADE 3.21 (http://www.ch.embnet.org/software/BOX_doc.html), with the fraction of sequences that must agree for shading set at 1.0. For the phylogeny, putative full-length land plant AtMYB93/92/53 homologues were identified using BLASTP from fully sequenced land plant genomes via GenBank and Phytozome (Goodstein et al., 2012). Sequences were aligned using CLUSTALX and the alignment was refined manually in SEA VIEW (Gouy et al., 2010). The phylogenetic tree was calculated using the maximum likelihood algorithm in SEA VIEW on default settings, with 1000 bootstrap replicates. Similar trees were obtained using distance methods. The tree was displayed using TREE VIEWX (Page, 2002).

Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh. Columbia (Col-0) ecotype was used. Atmyb92 (SM_3_41690), Atmyb93-1 (SALK_131752) and Atmyb93-2 (GK-588A05) insertion lines were obtained from the JIC-SM, SALK and GABI-KAT collections, respectively (Tissier et al., 1999; Alonso et al., 2003; Kleinboelting et al., 2012). The arabidillo1/2 double mutant was described previously (Coates et al., 2006), and was crossed with Atmyb93-1 to yield a triple arabidillo1/2/Atmyb93 mutant. Homozygous lines were identified by segregation analysis and by PCR/RT-PCR screening (Sessions et al., 2002).

Seeds were surface-sterilized using 20% Parozone™ (Jeyes, Cambridge, UK) and cold-treated (4°C) for 3 d before sowing. Seedlings were grown in sterile long-day conditions at 20–22°C on 0.5× Murashige and Skoog (MS) medium and 1% agar, pH 5.7, supplemented with hormones where required. Mature plants were grown in Levington M3 compost/vermiculite in the glasshouse (20–22°C, long days).

Root assays

Seedlings were grown vertically. To calculate emerged LR density in different genotypes (see the Results section, Figs 4c, 8g, S2c, 767) was cloned into pGBKT7 and used to screen a

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using a generalized likelihood test combined with a randomization procedure to generate $P$-values, in a manner analogous to methods for cDNA library comparison (Stekel et al., 2000; Herbert et al., 2008, 2011). For each strain comparison (wild-type versus mutant), the null hypothesis is that the frequency of LRPs at any given stage is the same between the two strains; the alternative hypothesis is that these frequencies are different. The log likelihood ratio of the observed frequencies under the two hypotheses was constructed using multinomial distributions to generate the test statistic. To generate a $P$-value, 10,000 simulated data sets were constructed using a multinomial distribution and the null hypothesis frequencies, and a test statistic was computed for each simulated data set. The $P$-value is approximated by the proportion of test statistics in the simulated data sets that are more extreme than the test statistic for the true data. Error bars were calculated using the standard error for a proportion, equal to $\sqrt{p(1-p)/n}$, where $p$ is the proportion and $n$ is the population size.

For hormone treatments (see the Results section, Fig. 7a–d), seedlings were grown for up to 12 d on 0.5× MS plates containing indole-3-acetic acid (IAA), abscisic acid (ABA) and naphthylphthalamic acid (NPA) or relevant solvent control, and emerged LR density was calculated as above. For statistical analysis, the null hypothesis that there is no difference between the behaviour of wild-type and that of Atmyb93 mutants under each treatment was tested using one-way analysis of variance (ANOVA) followed by a Tukey’s multiple comparison test.

For LRP induction experiments to assess the rate of LRP development, c. 20 seedlings per genotype were grown on vertical plates for 3 d before rotating the plate 90° to induce formation of a single LRP. Seedlings were cleared in Hoyer’s medium after either 18 or 42 h, and the stage of each induced LRP was scored at high magnification with a Leica DMRB microscope. For statistical analysis, a generalized likelihood test combined with a randomization procedure was applied as for the staging analysis as above.

Cloning and construct generation for transgenic plants

The full-length AtMYB93 promoter sequence (c. 1.6 kb upstream of the start codon) was amplified from Col-0 genomic DNA and cloned into pBI101 to make a pAtMYB93::GUS construct.

Fig. 1 The ARABIDILLO-1 ARMADILLO (ARM) domain interacts with three related R2R3 MYB family proteins: AtMYB92, -53 and -93. (a) Yeast two-hybrid interactions between the ARABIDILLO1 ARM-repeat domain and full-length Arabidopsis R2R3 MYB cDNAs expressed as GAL4-BD (GAL4-binding domain) and GAL4-AD (GAL4-activation domain) fusions, respectively. Growth on -LT (Leucine-Tryptophan) medium indicates successful co-transformation. Positive interactions are indicated by growth on -AHLT (Adenine-Histidine-Leucine-Tryptophan) medium and by blue colouration in the presence of Xα-gal. The ARABIDILLO-1 ARM-repeat domain interacts with AtMYB92, -53 and -93, but not with more distantly related AtMYB75/PAP1 (PAP1 = PRODUCTION OF ANTHOCYANIN PIGMENT 1) or AtMYB91/AS1 (AS1 = ASYMMETRIC LEAVES1). The interaction is specific to the C-terminus downstream of the R2R3 MYB domain. Conserved R2 and R3 MYB domains are shown in blue and magenta; the cyan box denotes the conserved C-terminal motif of AtMYB92, -53 and -93; the green box denotes a conserved C-terminal motif in AtMYB75/PAP1. (b) Co-immunoprecipitation of N-terminally HA-tagged AtMYB92 (MYC-MYB92; closed arrowhead) with the N-terminally MYC-tagged AtMYB92, -53 and -93. (a) Yeast two-hybrid interactions between the ARABIDILLO1 ARM-repeat domain and full-length Arabidopsis R2R3 MYB cDNAs expressed as GAL4-BD (GAL4-binding domain) and GAL4-AD (GAL4-activation domain) fusions, respectively. Growth on -LT (Leucine-Tryptophan) medium indicates successful co-transformation. Positive interactions are indicated by growth on -AHLT (Adenine-Histidine-Leucine-Tryptophan) medium and by blue colouration in the presence of Xα-gal. The ARABIDILLO-1 ARM-repeat domain interacts with AtMYB92, -53 and -93, but not with more distantly related AtMYB75/PAP1 (PAP1 = PRODUCTION OF ANTHOCYANIN PIGMENT 1) or AtMYB91/AS1 (AS1 = ASYMMETRIC LEAVES1). The interaction is specific to the C-terminus downstream of the R2R3 MYB domain. Conserved R2 and R3 MYB domains are shown in blue and magenta; the cyan box denotes the conserved C-terminal motif of AtMYB92, -53 and -93; the green box denotes a conserved C-terminal motif in AtMYB75/PAP1. (b) Co-immunoprecipitation of N-terminally MYC-tagged AtMYB92 (MYC-MYB92; closed arrowhead) with the N-terminally HA-tagged ARABIDILLO1 ARM domain (HA-ARM; open arrowhead). Proteins were synthesized in vitro, and co-incubated with anti-HA antibody. A control immunoprecipitation (IP) performed without the addition of HA-ARM was also conducted. I, input; W1/5, washes 1 and 5; E, elution; **, antibody heavy chain; *, nonspecific band in anti-MYC western blots. We performed similar experiments with AtMYB93, but because of the size of AtMYB93, it unfortunately could not be detected in the elution as it was obfuscated by the antibody heavy chain. (c) Alignment of the full-length amino acid sequences of AtMYB92, AtMYB53 and AtMYB93. Black and grey shading denotes identical and similar amino acid residues, respectively. Blue and magenta bars denote conserved R2 and R3 MYB domains, respectively. The cyan bar denotes the conserved C-terminal motif unique to these three proteins. *, key conserved aromatic residues within the R2R3 MYB domain.
 reporter. 35S::MYC-AtMYB93 and 35S::MYC-AtMYB93-YFP were constructed in pGreen0229 (Hellens et al., 2000). Constructs in Agrobacterium tumefaciens strain GV3101 were transformed into Arabidopsis by floral dip (Clough & Bent, 1998). Protoplast transfection was carried out as described previously (Nibau et al., 2011).

Promoter::GUS assays and imaging

Seedlings were assayed for β-glucuronidase activity according to standard protocols (Weigel & Glazebrook, 2002). Tissue was cleared through an ethanol/glycerol series and mounted in 50% glycerol for light microscopy. For hormone treatments, seedlings were grown vertically for 7 d before treatment with 1 μM IAA or 1 μM ABA in liquid 0.5× MS for 8 h and subsequent GUS staining. Sample preparation before confocal microscopy was carried out as described previously (Truermit et al., 2008). GUS/GFP imaging was carried out using Leica SP2 confocal microscopes.

RT-PCR and qRT-PCR

To test mRNA induction by phytohormones, 8-d-old seedlings were treated for 8 h in liquid 0.5× MS supplemented with IAA or ABA. To examine gene expression during LRP progression, LRs were induced using a gravity stimulus. Total RNA was extracted using an RNeasy plant mini kit (Qiagen, Venlo, Netherlands) and cDNA prepared using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) with oligo(dT) primers or random hexamer primers. qRT-PCR was carried out using the Sensimix™ kit (Quantace, London, UK) or the Sensimix™ kit (Bioline, London, UK) using 50 ng of cDNA template per reaction (endogenous control: ACTIN-2). Primers were designed using PrimerExpress software (ABI, Waltham, MA, USA). All PCRs were carried out using an ABI Prism 7000 instrument (Applied Biosystems, Waltham, MA, USA) or the LightCycler® 480 (Roche, Basel, Switzerland) with default thermocycling conditions. qRT-PCR results were analysed using the comparative C_T method (Schmittgen & Livak, 2008). Three biological replicates were carried out, each containing three technical replicates. To compare AtMYB93 expression levels in wild-type and Atmyb93-1, RNA extraction, cDNA synthesis and qRT-PCR were carried out as above on Col-0 and Atmyb93-1 root tissue. Two biological replicates were carried out, each containing four technical replicates. To present the data, the Col-0 expression level was set to 1.

Co-immunoprecipitation (coIP) experiments

The ARABIDILLO1 ARM domain (in pGBK7T7, which incorporates an N-terminal HA tag) and the full-length AtMYB92 (in pGADT7, which incorporates an N-terminal MYC tag) were translated in vitro using the TNT® T7 Coupled Reticulocyte Lysate System (Promega, Madison, WI, USA). Translated proteins (or nonprotein controls) were mixed and incubated in immunoprecipitation (IP) buffer (Nibau et al., 2011) at 4°C with rotation. HA-ARM was immunoprecipitated using EZ-View™ Red Anti-HA Affinity Gel (Sigma-Aldrich) and detected by western blot using anti-HA. Co-immunoprecipitated MYC-AtMYB92 was detected by anti-MYC western blot.

Western blotting

Seven-day-old seedlings were grown in liquid nitrogen and mixed with protein extraction buffer (125 mM Tris-HCl, pH 8.8, 1% SDS, 10% glycerol and 50 mM Na2S2O5) supplemented with protease inhibitor cocktail (Roche). For MG132 treatments, seedlings were preincubated for 2 h with MG132 or a dimethyl sulfoxide (DMSO) control. Equal protein amounts were resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA). Membranes were probed with primary antibodies: anti-MYC (Santa Cruz, Dallas, TX, USA), 1 : 1000; anti-α-tubulin (Sigma), 1 : 5000. Horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (Santa Cruz) was used at 1 : 10 000. Immunoblots were developed to film after using the ECL western blotting substrate (Pierce, Rockford, IL, USA).

Results

ARABIDILLO1 interacts with a specific group of related R2R3 MYB transcription factors

Previously we demonstrated that Arabidopsis ARABIDILLO proteins are positive regulators of LR initiation, containing an F-box, leucine-rich repeats and ARM repeats (Coates et al., 2006; Nibau et al., 2011). The full-length proteins are unstable, being turned over by the proteasome (Nibau et al., 2011). However, the ARABIDILLO1 ARM domain by itself is stable (Nibau et al., 2011), and ARM repeats are hypothesized to mediate protein–protein interactions (Coates, 2003). Thus, to understand the mechanism by which ARABIDILLO proteins function to promote LR development, we used the ARABIDILLO1 ARM domain as bait in a yeast two-hybrid screen of a seedling root cDNA library (Sorrell et al., 2003). We isolated a full-length clone of the R2R3 MYB transcription factor AtMYB92 (At5g10280) as a putative interaction partner of ARABIDILLO1 (Fig. 1a). To specify the region(s) of the AtMYB92 protein that interacts with the ARABIDILLO1 ARM domain, we tested truncated AtMYB92 constructs in the two-hybrid system. The C-terminus of AtMYB92 (AtMYB92C; amino acids 113–334), downstream of the R2R3 DNA-binding domain, interacts with the ARABIDILLO1 ARM, while the N-terminus (AtMYB92N; amino acids 1–112) does not (Fig. 1a). The interaction between the ARABIDILLO1 ARM and AtMYB92 was confirmed by co-immunoprecipitation (Fig. 1b).

AtMYB92 is one member of a small subfamily of Arabidopsis R2R3 MYB transcription factors, which also contains AtMYB93 (At1g34670) and AtMYB53 (At5g65230) (Kranz et al., 1998; Stracke et al., 2001). All three proteins share a conserved 41 amino acid motif downstream of the R2R3 MYB DNA-binding
Domain (26 amino acids longer than that identified by Kranz et al., 1998) that is not found in other Arabidopsis MYB proteins (Fig. 1c; Stracke et al., 2001; Dubos et al., 2010). Our alignments agree with previous phylogenetic analyses demonstrating that AtMYB92 and AtMYB53 are more similar to each other than either is to AtMYB93 (Fig. 1c; Stracke et al., 2001). To test whether these related proteins also interact with ARABIDILLO1, we cloned full-length AtMYB93 and AtMYB53 cDNAs. Both proteins interact with the ARABIDILLO1 ARM domain, while the more distantly related R2R3 MYB proteins AtMYB91 (AS1) and AtMYB75 (PAP1) do not (Fig. 1a).

The ARABIDILLO-interacting MYB subfamily is present only in flowering plants

As ARABIDILLO proteins are present in all land plants, including those without LRs (Nibau et al., 2011; Moody et al., 2012), we searched for putative ARABIDILLO-interacting MYB homologues throughout the plant lineage. We used the C-terminal regions of AtMYB93, AtMYB92 and AtMYB53 in BLASTP searches to identify full-length proteins in other species with similarity to the three ARABIDILLO-interacting MYBs across the entire length of their C-terminus. Putative homologues are present in both dicot and monocot flowering plants, which possess LRs, but are apparently absent from bryophytes (which lack true multicellular roots) and lycophytes (which lack LRs) (Fig. 2; alignment in Fig. S1). Thus, unlike ARABIDILLO proteins, which are found throughout the plant kingdom, putative homologues of ARABIDILLO-interacting MYB proteins are found only in flowering plants, which have multicellular, branched root systems that generate LRs.

AtMYB93 has root-specific expression and is up-regulated during LR development

To determine where AtMYB92, -93 and -95 genes are active, we examined their mRNA abundance in different plant tissues by semiquantitative RT-PCR (Fig. 3a). AtMYB92 and AtMYB53 mRNAs were detected throughout the plant but were enriched in roots, whereas AtMYB93 cDNA was amplified only from roots, indicating that its expression is restricted to this tissue (Fig. 3a).

Next we investigated whether these MYBs might play a specific role in LR development by examining their temporal expression by qRT-PCR in developing LRs that had been artificially induced by a gravitropic (bending) stimulus (Lucas et al., 2008; Peret et al., 2012). We detected an early increase in the mRNA levels of all three MYBs in ‘bend’ sections (excised from the PR) that were forming LRs (Fig. 3b), between 6 and 27 h after the gravitropic stimulus (from the first cell division to stage III in wild-type roots) (Peret et al., 2012). Following this initial up-regulation, levels decreased at later time-points (Fig. 3b). This increase was particularly striking for AtMYB93, which was up-regulated several thousand-fold, while the increases observed for AtMYB92 and AtMYB53 expression were >100- and 10-fold.

Fig. 2 AtMYB92, -93 and -95 homologues are found in flowering plants. An inferred phylogenetic tree of full-length AtMYB92, -93 and -95 protein homologues identified in the plant lineage from fully sequenced genomes is shown. Three main groups are identified: a group of dicot proteins most closely related to AtMYB92/AtMYB53 (black bar), a group of dicot proteins most closely related to AtMYB93 (dark grey bar) and a group of monocot proteins similar to AtMYB93 (light grey bar). The scale bar represents the average number of substitutions per column in the sequence alignment used for generating the phylogeny. Bootstrap values >0.5 are shown. At, Arabidopsis thaliana; Al, Arabidopsis lyrata; Br, Brassica rapa; Pt, Populus trichocarpa; Vv, Vitis vinifera; Lj, Lotus japonicus; Sb, Sorghum bicolor; Os, Oryza sativa.

Fig. 3 Arabidopsis AtMYB92, -93 and -95 expression is enriched in roots and induced in developing lateral root primordia (LRP). (a) Tissue-specific expression of AtMYB92, -93 and -95. AtMYB93 mRNA is detected exclusively in roots, while AtMYB92 and AtMYB53 mRNAs both show root-enriched expression. Data are representative of more than three biological replicates. (b) Relative AtMYB92 (white circles), -93 (grey circles) and -93 (black circles) gene expression over time in developing LRP (shown as log scale). mRNA abundance was analysed by qRT-PCR at the times indicated following initiation of LRP with a gravity stimulus. All three genes are up-regulated during early stages of LR development, and are down-regulated again at later time-points. This induction was particularly strong for AtMYB93 (>8000-fold increase by 27 h).
lower, respectively (Fig. 3b). The root-restricted localization of AtMYB93 expression, coupled with its very strong induction at sites where LRs are forming, suggested that AtMYB93 might function specifically during LR development.

AtMYB93 is a negative regulator of lateral root development

As AtMYB93 is the ARABIDILLO-interacting MYB most strongly and specifically expressed during LR development, we isolated a homozygous Atmyb93 T-DNA insertion mutant, Atmyb93-1, in which no AtMYB93 expression could be detected (Fig. 4a,b). When root growth and LR development were examined in detail, we found that the LR density (emerged LRs per cm of PR) was significantly higher in the Atmyb93-1 mutant than in wild-type plants (Fig. 4c). Emerged LR density was calculated for the entire length of the PR, for which no significant differences were observed between the genotypes (Fig. 4d). A second independent allele, Atmyb93-2, also showed a similar increase in emerged LR density (Fig. S2). We also obtained a homozygous T-DNA insertion in the AtMYB92 gene, but this mutant showed identical PR and LR morphology to wild-type (Fig. S3), suggesting either that AtMYB92 has no root function (possibly because of its much lower expression at sites of LR induction; Fig. 3b) or that it functions redundantly with AtMYB93. To test the latter possibility, we constructed an Atmyb92/Atmyb93-1 double mutant. This mutant showed a slightly, but significantly, stronger phenotype than the single Atmyb93-1 mutant (P<0.05 by ANOVA and Tukey’s multiple comparisons test; Fig. 4c), implying some (but not complete) overlap of function. We were unable

Fig. 4 Arabidopsis AtMYB93 is a negative regulator of lateral root development. (a) Intron–exon structure of the AtMYB93 gene coding region, showing the location of the Atmyb93-1 T-DNA insertion in exon 1. (b) qRT-PCR of AtMYB93 transcript in wild-type and Atmyb93-1 seedlings showing absence of AtMYB93 expression in the mutant relative to Columbia (Col-0). (c) Emerged lateral root (LR) densities in 10-d-old seedlings showing absence of AtMYB93 expression in the mutant relative to Col-0. (d) Primary root length (cm) in Atmyb93-1 and Atmyb92/Atmyb93-1 mutants have a greater LR density than wild-type plants. Two independent 35S::MYC::AtMYB93 lines have reduced LR density compared with wild-type plants. Data are from 10-d-old seedlings (n = 60 for all lines). (d) Primary root (PR) length is not significantly altered in Atmyb93-1 mutants compared with wild-type plants (n = 60). (e) Distribution of lateral root primordia (LRPs) at different developmental stages in 10-d-old seedlings of wild-type, Atmyb93-1 and Atmyb92/93-1 mutants and AtMYB93-overexpressing lines. Atmyb93-1 and Atmyb92/93-1 mutants have a larger proportion of LRPs at earlier stages (I–IV), indicating differences in the rate of LR development in the different mutant lines. P < 0.05 for wild-type versus 35S:AtMYB93 line 2 using a generalized likelihood test combined with a randomization procedure as described in the Materials and Methods section. (f, g) Percentage of LRPs at specific developmental stages 18 h (f) and 42 h (g) after induction of a single LRP per seedling using a gravity stimulus. Atmyb93-1 single and Atmyb92/Atmyb93-1 double mutants have faster LR initiation and LRP progression, as indicated by a larger proportion of induced primordia at later stages relative to wild-type, whereas 35S::MYC::AtMYB93 overexpression lines have slower LRP initiation and progression, as indicated by a larger proportion of primordia at earlier stages relative to wild-type (n = 20). All genotypes show a significant (P < 0.05) difference in distribution at 42 h using a generalized likelihood test combined with a randomization procedure as described in the Materials and Methods. Error bars (c, d), standard error of the mean. t-tests: *, P < 0.05; **, P < 0.01; ***, P < 0.001. Error bars (e–g), standard error of proportion.
to analyse a triple Atmyb mutant as no insertion lines for AtMYB53 are available. Next we generated transgenic Arabidopsis lines ectopically expressing MYC-tagged AtMYB93 driven by the cauliflower mosaic virus 35S (CaMV35S) promoter (35S::MYC-AtMYB93) and confirmed overexpression by immunoblotting (Fig. 8d). These AtMYB93-overexpressing lines displayed the opposite phenotype to the Atmyb93 loss-of-function mutants, namely a significantly reduced emerged LR density (Figs 4c, S2), and they also had a slight decrease in PR length (Fig. S4).

To further understand these altered LR densities in the mutants and overexpressing lines, we carried out a detailed LRP staging analysis for each genotype, examining the distribution of LRPs at each developmental stage along the full length of the root. Although there was no obvious build-up of LRPs at any particular stage that would indicate a major defect in the LRP emergence process, we found that the proportion of LRPs at late stages (VII and VIII (emerged)) was slightly higher in the Atmyb93 and Atmyb92myb93 mutants than in Col-0 (Fig. 4e). Moreover, the proportion of LRPs at early stages (I–IV) was greater in the AtMYB93-overexpressing lines than in Col-0 (Fig. 4e). These data suggested that there might be differences in the rate of LRP progression through development in the mutants and overexpressing lines, which would account for the differences in emerged LR densities.

To test this possibility further, we used a gravitropic stimulus to induce formation of a single LRP in multiple seedlings of each genotype (Lucas et al., 2008; Peret et al., 2012). The developmental stage of each induced LRP was then recorded within these seedling populations at both 18 and 42 h after applying the stimulus. We found that LRPs in the Atmyb93-1 and Atmyb92myb93-1 mutants progress through development faster than in wild-type, as indicated by a greater proportion of induced LRPs at later stages at both 18 and 42 h post-stimulus (Fig. 4f). By contrast, the speed of LRP progression in 35S::MYC-AtMYB93 seedlings was slightly delayed compared with wild-type, as the distribution of induced LRPs at both 18 and 42 h was shifted towards earlier stages compared with Col-0 (Fig. 4g).

Collectively, the increased speed of LR initiation and progression and emerged LR density of the Atmyb93 mutants, coupled with the opposite phenotypes of overexpression lines, indicate that AtMYB93 is a negative regulator of LR development.

**AtMYB93** gene expression is up-regulated by auxin specifically in the root basal meristem

LR development is regulated by many phytohormones (Nibau et al., 2008), and previous large-scale experiments have suggested that AtMYB93 is regulated specifically by both auxin and abscisic acid (ABA) (Kranz et al., 1998; Vanneste et al., 2005; Yanhui et al., 2006; Winter et al., 2007a; Lewis et al., 2013). To further our understanding of AtMYB93 gene regulation, we analysed the effects of both of these phytohormones on AtMYB93 gene expression in 8-d-old seedlings, using qRT-PCR. AtMYB93 gene expression was up-regulated (c. 2- to 3-fold) by both auxin (IAA) and ABA in a dose-dependent manner (Fig. 6a,b) but not by other hormones (gibberellin (GA), salicylic acid (SA) and jasmonic acid (JA)), corroborating previous findings ((Kranz et al., 1998; Yanhui et al., 2006; Winter et al., 2007) and data not shown). This is in contrast to both AtMYB92, which is not induced by IAA or ABA, and AtMYB53, which is induced by ABA but not IAA (Fig. 5; Kranz et al., 1998; Vanneste et al., 2005; Yanhui et al., 2006; Winter et al., 2007a; Lewis et al., 2013).

To validate the qRT-PCR data, and localize where AtMYB93 induction was occurring in the root, we analysed AtMYB93 promoter activity in response to auxin and ABA using pAtMYB93::GUS. Hormone application did not lead to significant temporal or spatial changes in the expression of pAtMYB93::GUS in the endodermal cells overlying LRPs (Fig. 6c). However, upon exogenous auxin application, additional weak pAtMYB93::GUS expression was detected specifically in the basal meristem of the PR (Fig. 6d), a region instrumental in determining the position and spacing of LRPs (De Smet et al., 2007). In response to ABA, no change in the intensity or pattern of pAtMYB93::GUS was seen either in the basal meristem (Fig. 6d) or in the rest of the plant. This suggests a different mode of regulation for AtMYB93 gene expression in response to auxin compared with ABA. The spatial specificity of AtMYB93s auxin induction suggests that the inhibitory role of AtMYB93 during LR initiation is linked to auxin signalling, and may occur very early during
LRP/pericycle cell priming in the basal meristem (De Smet et al., 2007).

Atmyb93 mutants show reduced sensitivity to auxin during lateral root development

As AtMYB93 is an LR inhibitor up-regulated by both auxin and ABA, we tested the sensitivity of Atmyb93-1 mutant LR development to both hormones. The concentrations of auxin (IAA) tested inhibit PR elongation and promote the initiation stage of LR development (Blakely et al., 1988; Laskowski et al., 1995; Coates et al., 2006; Ivanchenko et al., 2010). Atmyb93-1 mutants responded to IAA similarly to wild-type with respect to PR elongation (Fig. 7a), but showed insensitivity to LR induction by exogenous IAA when applied at a concentration of 1 μM (Fig. 7b). No significant insensitivity of Atmyb93-1 mutants was seen when using lower concentrations of auxin (25–500 nM) (data not shown).

As LR initiation and emergence both require auxin transport (Reed et al., 1998; Bhalerao et al., 2002), we tested the response of Atmyb93-1 to the auxin transport inhibitor NPA. NPA inhibited LR development in the Atmyb93-1 mutant similarly to in wild-type plants (Fig. 7c). Moreover, Atmyb93-1 seedlings treated with NPA and then transferred to normal growth medium still formed more LRs than similarly treated wild-type seedlings (Fig. S6). This suggests that AtMYB93 does not repress LR development via auxin transport pathways, but instead negatively affects auxin signalling. ABA inhibits LR emergence after the initiation stage, at concentrations (< 1 μM) that do not affect PR growth (De Smet et al., 2003). Atmyb93-1 mutants responded as wild-type to ABA, showing a marked decrease in the number of emerged LRs present upon ABA treatment (Fig. 7d).

Thus, Atmyb93-1 mutants are somewhat insensitive to auxin, specifically with respect to LR development, but show normal responses to auxin transport inhibitors and ABA. Collectively, these data suggest that AtMYB93 is an LR-specific modulator.

**Fig. 5** AtMYB93 promoter activity is restricted to endodermal cells overlying early-stage lateral root primordia in Arabidopsis. (a) pAtMYB93::GUS expression in the mature root is restricted to sites of developing lateral root primordia (LRPs) (arrowheads). (b) pAtMYB93::GUS expression is absent from the cotyledons and hypocotyls of seedlings. (c) pAtMYB93::GUS expression is absent from the primary root (PR) tip. (d) Detailed expression analysis of pAtMYB93::GUS in developing LRP. GUS activity in the endodermis is detected before the first asymmetric division, and throughout early stages (I–V) of development. During later stages (VI–VII), GUS activity fades and is completely absent upon emergence. Arrowheads show early cell divisions. (e) Confocal image of an early-stage LRP (asterisk) showing that pAtMYB93::GUS (green) is expressed exclusively in the endodermal layer overlying the primordia (arrowhead). P, pericycle; En, endodermis; C, cortex; Ep, epidermis. Cell walls are stained with propidium iodide. (f, g) Confocal images of LRPs showing that pAtMYB93::GUS expression is localized to the cells surrounding primordia as they develop through later stages. Cell walls are stained with propidium iodide. Bars: (a, c–e) 50 μm; (b) 200 μm.
required for normal auxin-signalling responses during LR development, and therefore represents a novel auxin-induced negative regulator of LR development.

AtMYB93 is not a degradation target of ARABIDILLOs

We identified AtMYB93 as an interaction partner of the ARABIDILLO1 ARM domain in yeast. ARABIDILLO1 is an F-box protein proposed to facilitate ubiquitination and degradation of target protein partners, and arabidillo1/2 double mutants have reduced LR densities (Nibau et al., 2011). Given the opposite phenotypes of arabidillo and Atmyb93 loss-of-function mutants, one hypothesis is that ARABIDILLO proteins target AtMYB93 for degradation.

To test this possibility, we generated a MYC-tagged AMyB93-YFP fusion protein driven from the CaMV35S promoter, which localized to both the nucleus and the cytosol of Arabidopsis protoplasts and stably transformed wild-type and arabidillo1/2 mutant seedlings (Fig. 8a,b). The relative abundance of MYC-AtMYB93-YFP was not enhanced in the arabidillo1/2 mutant, suggesting that ARABIDILLOs do not regulate AtMYB93 stability (Fig. 8b). To confirm this, we compared the protein levels of 35S::MYC-AtMYB93-YFP in wild-type and arabidillo1/2 mutant seedlings by western blotting and detected no difference in protein abundance (Fig. 8c). Moreover, treatment of transgenic 35S::MYC-AtMYB93 seedlings with the proteasome inhibitor MG132 did not lead to an accumulation of the MYC-AtMYB93 protein (Fig. 8d), suggesting that global AtMYB93 stability is regulated neither by ARABIDILLOs nor by general proteasomal turnover. Furthermore, there was no change in AtMYB93 mRNA levels in an arabidillo1/2 background, and no change in arabidillo mRNA levels in an AtMYB93-1 mutant.
background (Fig. S7), implying that the AtMYB93 and ARABIDILLO genes do not affect each other’s transcription. This suggests that the AtMYB93–ARABIDILLO interaction may control LR development via a nonproteasomal mechanism. To test this suggestion genetically, we generated an arabidillo1/2/Atmyb93-1 triple mutant, which showed reduced LR density, similarly to the arabidillo1/2 mutant (Fig. 8e). This result corroborates our finding that ARABIDILLOs do not degrade AtMYB93, and suggests instead that an ARABIDILLO-mediated promotion of LR development in wild-type plants can be repressed by AtMYB93 interacting with ARABIDILLOs. Thus, AtMYB93 may integrate hormonal responses to modulate ARABIDILLO-mediated LR promotion.

**Discussion**

**AtMYB93 functions to inhibit LR development in Arabidopsis**

AtMYB93 is expressed transiently at sites of early LR development, specifically in the endodermal cells overlying developing LRPs, and is also induced by auxin in the basal meristem of the PR (a region where the patterning and spacing of LR initiation sites are regulated). AtMYB93 loss of function is sufficient to cause an increase in the rate of LRP developmental progression and thus an enhanced LR density, while overexpression of AtMYB93 decreases LR progression and subsequent emerged LR density. These data therefore suggest that AtMYB93 is an early-induced inhibitor of LR development.

The enhanced LR density of the Atmyb93 mutant is quite subtle and contrasts with the zero or severely reduced LR phenotypes seen in other well-characterized LR mutants affected in the early stages of LR development (such as iaa14slr1, arf7arf19, iaa28 and gata23 (Rogg et al., 2001; Fukaki et al., 2005; Okushima et al., 2005; De Rybel et al., 2010; reviewed in Peret et al., 2009a). This suggests a modulatory role for AtMYB93 in LR development. We propose that the LR initiation process includes the early induction of an AtMYB93-dependent negative feedback module capable of repressing LR development under certain conditions (e.g., stress, or changes in the nutrient status of the plant), and which is necessary for normal LR development. Future genetic analyses will allow the functional relationship between AtMYB93 and known key positive regulators of LR initiation to be established.

The observation that the AtMYB93 promoter is active in the endodermal cells overlying developing LRPs, rather than in the primordia themselves, implies that the overlying tissues...
contribute to AtMYB93-mediated negative feedback. This cell-type-specific localization also corroborates the findings of previous studies showing that AtMYB93 is a target of the endodermal transcription factor SCARECROW (SCR) (Iyer-Pascuzzi et al., 2011) and that AtMYB93 mRNAs are significantly up-regulated in the endodermis-specific translatome (Mustroph et al., 2009). It was recently shown that the endodermis regulates LR initiation: PIN3 (PIN-FORMED3)-dependent auxin movement between the endodermis and the pericycle acts as a ‘checkpoint’ for initiation (Marhavy et al., 2013), while endodermal cells change morphology very early during the LR developmental process to accommodate pericycle cell expansion and division (Vermeer et al., 2014). It will be interesting to determine whether AtMYB93 activity is linked to either of these processes. The AtMYB93 protein may be active in the endodermis, perhaps sending a signal to the pericycle cells as LR initiation commences, or contributing to the regulation of the remodeling or separation of overlying tissues that is required for very early LRP progression (Peret et al., 2009b; Vermeer et al., 2014). Alternatively, the AtMYB93 protein may act cell nonautonomously, moving into the pericycle or early LRP s, a phenomenon observed for other key regulatory transcription factors (Nakajima et al., 2001; Schlereth et al., 2010). Future analysis of AtMYB93 protein localization in relation to its activity will address all of these possibilities and provide mechanistic insight into AtMYB93 function during LR development.

Interaction of AtMYB93 with auxin and ABA signalling

In addition to stimulation by root bending, AtMYB93 expression is up-regulated by auxin and ABA, two key phytohormones that regulate LR development in a complex manner (De Smet et al., 2003; Liang et al., 2007; Peret et al., 2009a,b; Ivanchenko et al., 2010). Auxin-induced up-regulation of pAtMYB93::GUS occurs specifically in the root basal meristem, a region where oscillating auxin sensitivity and a recurrent auxin signal determine the future position and spacing of LRs originating from the pericycle (De Smet et al., 2007; De Rybel et al., 2010; Moreno-Risueno et al., 2010). This suggests that AtMYB93 may have a very early auxin-related function during LR initiation. The presence of an auxin-induced LR repressor such as AtMYB93 in the basal meristem might help to ensure the regularity and robustness of auxin oscillation. De Smet et al. (2007) postulated the existence of an additional auxin response module(s) required for LR development, including an attenuation signal that ensures that LR initiation only occurs at one xylem pole at a time. It is tempting to speculate that AtMYB93 may be involved in this process: no other candidates for an attenuation signal have been proposed to date. It is interesting to note that AtMYB93 up-regulation by auxin is blocked in arf7 and arf7/19 mutants (Okushima et al., 2005), suggesting that AtMYB93 functions downstream of (and could indeed be induced by) the first auxin signalling module in the basal meristem that controls LR initiation (De Rybel et al., 2010).

Our data suggest that AtMYB93 is required for normal auxin signalling specifically during LR development, as the insensitivity of the Atmyb93 mutant to higher concentrations of exogenous auxin is only observed in LRs and not in the PR. This identifies AtMYB93 as the first known auxin-induced negative regulator specifically involved in very early LR development. The only previously identified auxin-induced negative regulator of LR development is the pleiotropic-functioning auxin signalling protein SHY2 (SHORT HYPOCOTYL2); however, SHY2 only inhibits the later stages of LR development and is in fact a positive regulator of initiation events (Tian & Reed, 1999; Swarup et al., 2008; Goh et al., 2012). It may seem surprising that Atmyb93 mutants are insensitive to higher concentrations of exogenously applied auxin, given that AtMYB93 is an auxin-induced negative regulator of LRs. However, we suggest that if normal LR development requires a functional negative feedback loop, then loss of the feedback loop may block further auxin-induced LR development. The fact that Atmyb93 mutants are neither insensitive nor hypersensitive to concentrations of exogenous auxin below 1 μM confirms that additional AtMYB93-independent feedback mechanisms (for example, a SHY2 module) are also likely to regulate normal LR development.

In addition to auxin, AtMYB93 gene expression is induced by exogenous ABA. Atmyb93 mutant LRs respond to ABA similarly to wild-type and no additional induction of pAtMYB93::GUS by ABA is seen in the seedling root, suggesting that ABA does not play a role in the control of LR development by AtMYB93. We cannot rule out the possibility that ABA plays an additional regulatory role(s) under certain conditions or stresses, or at other stages in the plant life cycle. Interestingly, AtMYB93 is a direct transcriptional target of SCR, which defines the cell identity of the root endodermis and cortex, regulates ABA-mediated germination and is differentially regulated in the root by various abiotic stresses (Iyer-Pascuzzi et al., 2011). Moreover, the endodermis regulates later LR developmental progression in response to salt stress (Duan et al., 2013), and LR responses to environmental stress differ from PR responses (Tian et al., 2014), suggesting that AtMYB93, which has an LR-specific function, could also have stress-responsive roles. This will be addressed in future studies.

AtMYB93 is part of a small gene family in Arabidopsis with relatives in other flowering plants

AtMYB93 is one of three related Arabidopsis ARABIDILLO1-interacting R2R3 MYB genes and is the only member of its subclade with root-restricted expression. We have shown that AtMYB93 is also the only auxin-responsive member of its subclade, corroborating the findings of previous large-scale and LR-specific transcriptional studies (this work; Kranz et al., 1998; Okushima et al., 2005; Yanhui et al., 2006; Winter et al., 2007; Lewis et al., 2013). As AtMYB93 is divergent from AtMYB92 and AtMYB53, we suggest that AtMYB93 may have acquired novel root-regulating functions during evolution. AtMYB93 functions only partially redundantly with its relative(s), as the Atmyb92 mutant has no LR phenotype. AtMYB92 and AtMYB53 are expressed throughout the plant: future studies will address the functions of these genes during plant development.

Homologues of AtMYB93 and its relatives are present in flowering plants but appear to be absent from plants lacking LRs,
namely bryophytes and lycophytes. This is in contrast to ARABIDILLO proteins, which are very highly conserved across all land plants (Nibau et al., 2011; Moody et al., 2012). It seems likely that ARABIDILLO proteins evolved an LR-promoting function either after the divergence of the flowering plant lineage or after the evolution of true LRs. Identification of AtMYB93 homologues in gymnosperms and ferns would help to resolve these scenarios. We cannot currently distinguish between the possibilities that (i) early-evolving ARABIDILLO proteins could interact with more divergent MYBs in early land plants and perform nonroot functions and (ii) early-evolving ARABIDILLOs could have nonMYB interaction partners.

How does AtMYB93 functionally interact with ARABIDILLO proteins?

AtMYB93 and its close homologues in Arabidopsis interact with ARABIDILLO1, a positive regulator of LR initiation (Coates et al., 2006). Given that ARABIDILLO proteins are F-box proteins that associate with the SKP1 (S-phase kinase-associated protein 1) component of the proteasomal degradation machinery, our previous hypothesis was that ARABIDILLO proteins target an inhibitor of LR formation, such as AtMYB93, for degradation (Nibau et al., 2011). However, our experiments have not detected any regulation of AtMYB93 stability by ARABIDILLO proteins. Moreover, we previously showed that mutating key F-box residues in ARABIDILLO1 does not abolish the protein’s in planta function during LR formation, indicating that its function during the promotion of LR development is not linked to its putative role as an E3 ligase (Nibau et al., 2011). Thus, an alternative possibility is that ARABIDILLO and MYB proteins could interact to form a functional protein complex that regulates the transcription of downstream genes, with ARABIDILLOs promoting LR development and AtMYB93 binding as a repressor. This scenario is similar to what is seen with animal beta-catenin/Armadillo proteins (to which ARABIDILLOs are structurally related), which interact with both transcriptional activators and repressors (Valenta et al., 2012). The arabadillo-like phenotype of the arabadillo1/arabadillo2/Atmyb93 triple mutant supports this possibility. We suggest that, in the absence of AtMYB93 (the Atmyb93 single mutant), ARABIDILLOs can promote LR development to a greater extent than in wild-type plants, whereas in the absence of ARABIDILLO proteins the promotion of LR development can no longer occur, regardless of the presence of AtMYB93. The functional mechanism of the interaction between AtMYB93 and ARABIDILLO will be the target of future study. Furthermore, future focus will be placed on identifying ARABIDILLO and AtMYB93 gene targets and uncovering how these modifiers of LR development interact with the myriad other signalling components that regulate this complex and highly plastic developmental process.

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References


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Supporting Information
Additional supporting information may be found in the online version of this article.

Fig. S1 Protein sequence alignment of AtMYB93, -92 and -53 homologues in land plants.

Fig. S2 A second Atmyb93 allele, Atmyb93-2, shows increased LR formation.

Fig. S3 An Atmyb92 single mutant has no PR or LR phenotypes.

Fig. S4 PR length is slightly reduced relative to wild-type in 35S::MYC-AtMYB93 seedlings.

Fig. S5 qRT-PCR analysis of the effect of exogenous IAA and ABA on MYB92 and MYB53 expression.

Fig. S6 Atmyb93 mutants grown on NPA for 7 d and then transferred to 0.5× MS are still able to produce more lateral roots than wild-type plants.

Fig. S7 qRT-PCR analysis of ARABIDILLO1 and AtMYB genes in arabidillo1/2, Atmyb93 and Atmyb92/Atmyb93 mutants.

Table S1 List of primers used in this work

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