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Diversification of a Transcription Factor Family Led to the Evolution of Antagonistically Acting Genetic Regulators of Root Hair Growth

Highlights

- The LRL bHLH transcription factor family diversified during land plant evolution
- Expression of individual *LRL* genes was gradually restricted to specific domains
- In derived lineages, there are two sets of antagonistically acting *LRL* genes
- *LRL* gene function is partially conserved between streptophyte algae and angiosperms

Authors

Holger Breuninger, Anna Thamm, Susanna Streubel, Hidetoshi Sakayama, Tomoaki Nishiyama, Liam Dolan

Correspondence

liam.dolan@plants.ox.ac.uk

In Brief

Breuninger et al. show that the LRL transcription factor family of angiosperms is derived from a single-copy gene in early diverging plant lineages. The single-copy Mp*LRL* gene acts as a general growth regulator in liverworts. LRL function diversified into two antagonistically acting groups of proteins active in root hair development in angiosperms.

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Diversification of a Transcription Factor Family Led to the Evolution of Antagonistically Acting Genetic Regulators of Root Hair Growth

Holger Breuninger,¹ Anna Thamm,¹ Susanna Streubel,¹ Hidetoshi Sakayama,² Tomoaki Nishiyama,³ and Liam Dolan^{1,*}

¹Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK

²Department of Biology, Graduate School of Science, Kobe University, 1-1 Rokkodai, Nada-ku, Kobe 657-8501, Japan

³Advanced Science Research Center, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-0934, Japan

*Correspondence: liam.dolan@plants.ox.ac.uk

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SUMMARY

Streptophytes colonized the land some time before 470 million years ago [1–3]. The colonization coincided with an increase in morphological and cellular diversity [4–7]. This increase in diversity is correlated with a proliferation in transcription factors encoded in genomes [8–10]. This suggests that gene duplication and subsequent diversification of function was instrumental in the generation of land plant diversity. Here, we investigate the diversification of the streptophyte-specific *Lotus japonicus* ROTHAIRLESS LIKE (LRL) transcription factor (TF) [11, 12] subfamily of basic loop helix (bHLH) proteins by comparing gene function in early divergent and derived land plant species. We report that the single *Marchantia polymorpha* LRL gene acts as a general growth regulator required for rhizoid development, a function that has been partially conserved throughout multicellular streptophytes. In contrast, the five relatively derived *Arabidopsis thaliana* LRL genes comprise two antagonistically acting groups of differentially expressed genes. The diversification of LRL genes accompanied the evolution of an antagonistic regulatory element controlling root hair development.

RESULTS AND DISCUSSION

LRL Gene Number Increased in Land Plants

To identify LRL-related (group XI basic helix loop helix) gene sequences from early diverging land plants and streptophyte algae, we performed BLAST searches on *Marchantia polymorpha* and *Chara braunii* sequences using *Arabidopsis thaliana* AtLRL sequences as queries. Single transcripts, Cb_bHLHtranscript1 (GenBank accession number KX037431) and Mp_bHLHtranscript1 (KX037432), were identified in each species encoding proteins with basic loop helix (bHLH) and LRL domains similar to those found in PpLRL1 of *Physcomitrella patens* and AtLRL3 of *A. thaliana* (Figure S1) and were therefore designated MpLRL and CbLRL, respectively. These genes are most likely sin-

gle-copy genes in *C. braunii* and *M. polymorpha* because a single band was detected in Southern blots of gDNA hybridized with a probe containing the LRL domain sequence (Figure S1). In flowering land plants, LRL transcription factors (TFs) form three well-supported clades (XIa, XIb, and XIc, termed here class I, II, and III [12]). Comparison of MpLRL with other LRL sequences demonstrated that MpLRL and PpLRL are placed outside these three clades suggesting that LRL TFs diversified after the divergence of bryophytes and flowering plants (Figure S1). The CbLRL is placed among the class I LRL sequences on a long branch (Figure S1). No LRL-related sequences were identified in *Chlamydomonas reinhardtii* or *Ostreococcus tauri* or in the filamentous streptophyte *Klebsormidium flaccidum* suggesting that LRL TFs evolved among complex streptophyte algae. Taken together, these data indicate that the LRL proteins constitute a highly conserved transcription factor family, which diversified during streptophyte evolution from a most likely single-copy gene in the most ancestral groups of Charales and liverworts to a gene family with several members belonging to different monophyletic clades in flowering plants.

LRL Gene Expression Is Restricted to Specific Domains in *Arabidopsis*

To assess whether diversification of the LRL gene family was accompanied by changes in gene expression, we compared expression patterns of LRL genes in *C. braunii*, *M. polymorpha* and *A. thaliana*. In situ hybridization in *C. braunii* showed that LRL mRNA was present in most tissues of the plant; CbLRL mRNA was detected in the nodes, internodes, and the oogonium surrounding the egg cell and zygotes (Figures 1A, 1G, and S1). In *M. polymorpha*, highest levels of MpLRL mRNA were detected in the meristematic notch by in situ hybridization (Figures 1B, 1C, and S1). Highest fluorescence levels in the meristematic notch was observed in plants transformed with *proMpLRL:NLS-3xYFP* (Figures 1D, 1H, and 1I) indicating that the promoter was most strongly active in the same cells in which the mRNA was detected. Lower levels of YFP fluorescence were detected throughout the thallus (Figures 1E, 1J, 1F, 1K, and S1). The relatively high expression in the notch and lower expression in the thallus is different from what is observed with ubiquitously expressed reporters (compare Figure 1D with Figure S1J), indicating that the MpLRL expression heterogeneity is not caused by differences in cell densities along the thallus. Together, these data indicate that MpLRL is more highly expressed in the vicinity of the meristem than elsewhere in the thallus. This preferential

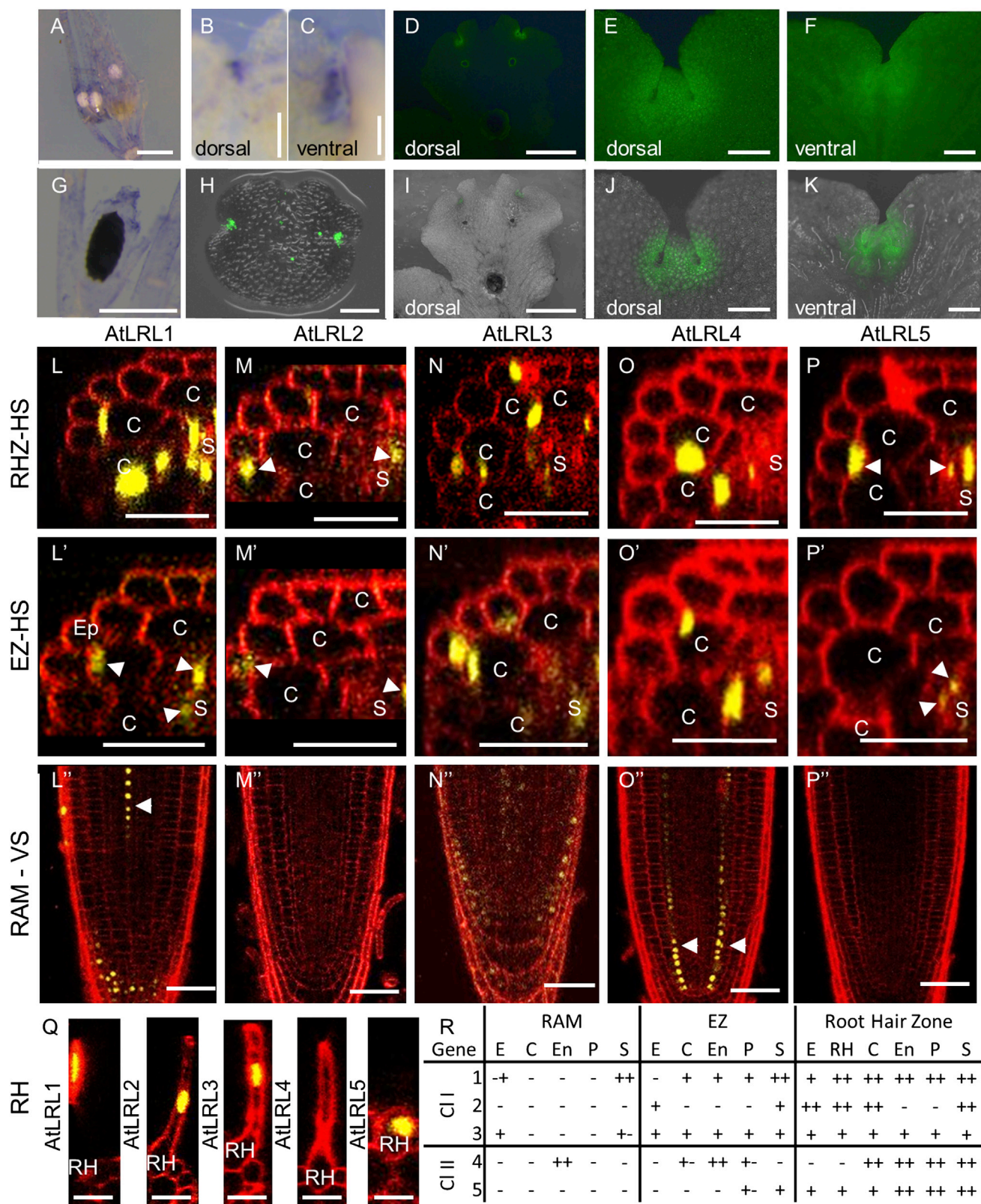


Figure 1. The LRL Expression Pattern Diversifies into Specific Domains

(A and G) Whole-mount in situ of *CbLRL*. Gene expression was detected in all tissue types analyzed. (A) Part of thallus consisting of a main axis and whorled branchlets with reproductive organs and oogonium with unfertilized egg cell. (G) Fertilized zygote surrounded by oogonial tube cells. Scale bars, 1 mm (A) and 100 μ m (G).

(legend continued on next page)

expression in some domains is also observed in another early diverging land plant, the moss *P. patens* [12].

We determined the spatial expression pattern of each of the five *A. thaliana* *LRL* genes using promoter:YFP fusions. The fluorescence patterns revealed that each of the promoters is active in distinct though overlapping domains. Summed together these expression domains include cells of most tissue types (Figure S2; summary in Figure 1R). In the root, the differences between the *AtLRL* gene expression patterns were most pronounced in the root apical meristem (RAM) and elongation zone (EZ; Figures 1L'–1P''). The promoters of *AtLRL1* and *AtLRL4* were active early in the development of the provascular tissue and endodermis, respectively (Figures 1L'', 1L', O'', and O'). *proAtLRL3* was active in every cell and tissue (Figures 1N'' and 1N'); neither *proAtLRL2* nor *proAtLRL5* were active in the RAM (Figures 1M'' and 1P''). Later, in the root-hair zone (RHZ), *proAtLRL1* and *proAtLRL3* were active in every cell, while the *proAtLRL2* was active in most cells except pericycle and endodermis (Figures 1L–1N and 1Q). *proAtLRL4* was active in all tissues except the epidermis in the root hair zone, whereas *proAtLRL5* activity was detected in all tissues (Figures 1O–1Q). Taken together, these data indicate that single-copy *LRL* genes are broadly expressed in *C. braunii* and *M. polymorpha* plants, while the different *A. thaliana* *LRL* genes are expressed in distinct but overlapping domains. This suggests that *LRL* genes function throughout the plant in streptophyte algae and became progressively more restricted to specific tissues in land plants. In derived land plant taxa, multiple *LRL* genes are expressed in distinct domains, suggesting that different genes have distinct though overlapping functions.

MpLRL Function Is Required for Dorsal Thallus and Rhizoid Development

To investigate whether changes in gene expression patterns reflect changes in gene function, we compared *M. polymorpha* and *A. thaliana* *LRL* gene function. We generated two different inducible artificial microRNA (amiR) constructs to reduce MpLRL activity [13] using the ethanol inducible AlcR/AlcA system [14] in which the induced expression could be monitored with an inducible *AlcA:NLS-3xCFP* reporter on the same T-DNA. This construct, *AlcA:amiR-MpLRL^{Mpmir160}-AlcA:NLS-3xCFP-proOsAct:AlcR* (the expression pattern of *proOsAct* is shown in Figure S1), was transformed and two lines for each *amiR-MpLRL^{Mpmir160}* sequence were established—*amiRI-MpLRL^{Mpmir160}-ind-1* and *amiRI-MpLRL^{Mpmir160}-ind-2*; *amiRII-*

MpLRL^{Mpmir160}-ind-1 and *amiRII-MpLRL^{Mpmir160}-ind-2*. Each line expressed CFP and developed a defective phenotype only upon ethanol induction (20% ethanol vapor; Figure S3). Induction of the expression of the *amiR-MpLRL^{Mpmir160}* microRNAs in these lines caused growth defects in gemmae—vegetative propagules of *M. polymorpha*—which we classified into five categories (C1–C5): (C1) wild-type like plants; (C2) plants with undifferentiated patches of tissue outgrowth on the dorsal thallus; (C3) plants with larger outgrowths on the dorsal thallus indicating the initiation of secondary, ectopic thallus (Figure 2B); (C4) plants with a fully grown ectopic secondary thallus (Figure 2C); and (C5) the entire dorsal side of the plant was covered with rhizoid-less callus like tissue (Figure 2D). Using these categories, we quantified the phenotypic variation in one *amiRI-MpLRL^{Mpmir160}-ind-1* and one *amiRII-MpLRL^{Mpmir160}-ind-1* line. Both lines developed a significant number of callus-like phenotypes (C5 phenotype), but the frequency of the phenotypes depended on the *amiR-MpLRL^{Mpmir160}* sequences used (Figure S3). *amiRI-MpLRL^{Mpmir160}-ind-1* plants generally form secondary thalli (C4 phenotype), while up to 46.9% of meristems in lines carrying the *amiRII-MpLRL^{Mpmir160}-ind-1* construct developed callus like tissue (C5 phenotype). All defects could be complemented by double-transforming the *amiRII-MpLRL^{Mpmir160}-ind-1* line with an *amiRII-MpLRL^{Mpmir160}*-resistant MpLRL cDNA, *MpLRLres*, driven by the constitutive *proOsAct* promoter. The majority of meristems that developed on two independent MpLRL knockdown lines transformed with the *MpLRLres* gene developed wild-type features (compare Figure 2F with Figures 2G and 2H). To independently determine the function of MpLRL, we fused the MpLRL cDNA to the EAR repressor domain SRDX [15, 16] and expressed this fusion under the control of *proOsAct* promoter. The growth of the dorsal thallus of *proOsAct:MpLRL-SRDX* plants was severely impaired, resulting in the development of plants without dorsal characteristics and prominent ventral scales without rhizoids (Figures 2K, 2L, and S3). Severe morphological defects developed in 2-week-old gemma expressing the MpLRL-SRDX fusion using an ethanol inducible MpLRL-SRDX gene construct (compare MpLRL-SRDXind-1 and MpLRL-SRDXind-2 in Figure S3). These plants were morphologically similar to the severe C4 and C5 phenotypes observed in *amiRII-MpLRL^{Mpmir160}-ind-1* lines. Taken together, the absence of rhizoids in the most severe phenotypes of the *amiR-MpLRL^{Mpmir160}* and *MpLRL-SRDX* lines indicates that MpLRL positively regulates rhizoid development. These results indicate that MpLRL is a general growth regulator and required for dorsal development in gemmae.

(B and C) Whole-mount in situ hybridization of MpLRL. Strongest gene expression was detected in meristematic tissues. (B) Dorsal view; (C) ventral view. Scale bars, 100 μ m.

(D and I) Dorsal view of a *M. polymorpha* thallus expressing *proMpLRL:NLS-3xYFP*. (D) YFP fluorescence of dorsal thallus. (I) Overlay of bright-field and YFP fluorescence. Note the higher fluorescence signal in the meristematic notch. Scale bars, 5 mm.

(E, F, J, and K) Close-up view of a meristematic notch in *M. polymorpha* expressing *proMpLRL:NLS-3xYFP* on dorsal and ventral side, respectively. (E and F) YFP signal only and (J and K) showing the overlay of YFP fluorescence and bright field. Note the relatively weaker signal outside of the meristematic notch. Scale bars, 500 μ m.

(H) Overlay of bright-field image and YFP fluorescence of *M. polymorpha* gemma expressing *proMpLRL:NLS-3xYFP*. Scale bar, 100 μ m.

(L–P'') Confocal images of *proAtLRL1* (L), *proAtLRL2* (M), *proAtLRL3* (N), *proAtLRL4* (O), and *proAtLRL5* (P) promoter fusions to NLS-3xYFP within the root hair zone (RHZ), elongation zone (EZ; L'–P'), and the root apical meristem (RAM; L''–P''). VS, vertical section; HS, horizontal section.

(Q) Root hair expression of *AtLRL* genes. Only the promoter of *AtLRL4* was not detected in root hair cells (RH).

(R) Table summarizing *AtLRL* expression domains in the root. + and – indicate expression with –, negative; ++, strong. E, epidermis; RH, root hair; C, cortex; En, endodermis; P, pericycle; S, stele.

See also Figures S1 and S2.

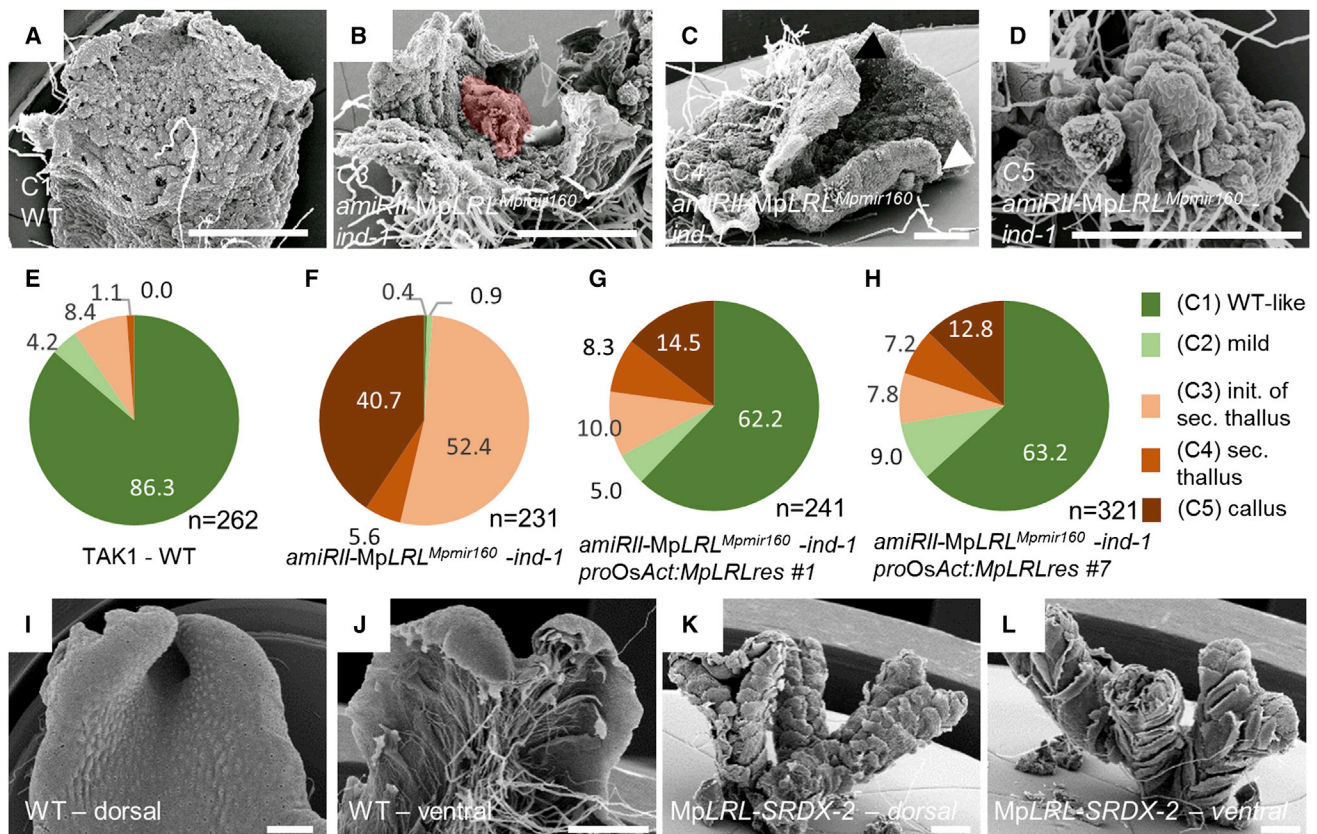


Figure 2. MpLRL Is a Growth Regulator Required for Both Dorsal Thallus and Rhizoid Development

(A–D) Phenotypes of plants grown from gemmae transformed with the inducible *MpLRLamiRIL^{Mpmir160}* transgene in inductive conditions compared to wild-type. (A) Wild-type plants with category (C1) phenotype, (B) plants with initiating outgrowth (colored patch) on the dorsal thallus indicative for an outgrowing thallus (C3), (C) plants with secondary thallus formed on the dorsal side (C4, arrowheads indicate meristems: white arrow, primary thallus; black arrow, secondary thallus), and (D) plants with only rhizoid-less callus like tissue developing on the dorsal thallus (C5).

(E and F) Quantification of meristematic phenotypes of *MpLRLamiRIL-ind-1* grown under inductive conditions. (E) Wild-type and (F) *MpLRLamiRIL-ind-1*.

(G and H) Quantification of meristematic phenotypes of two independent double-transformants of *proOsAct:MpLRLres* in the *MpLRLamiRIL-ind-1*. Transformant line #1 (G) and transformant line #7 (H). Note the large number of relatively wild-type-like meristems and the small number of the strong, category C5 phenotypes. (I and J) Fully grown wild-type *M. polymorpha* thalli. (I) Dorsal view and (J) ventral view are shown.

(K and L) Fully grown *M. polymorpha* thalli transformed with the *proOsAct:MpLRL-SRDX* fusion. (K) Dorsal view; (L) ventral view. Note the lack of dorsal-like tissue types in (K) and the lack of rhizoids in (L).

Scale bars, 500 μ m. See also Figure S3.

The Two Classes of AtLRL Genes Act Antagonistically

To define the function of *LRL* genes in angiosperms, we generated gain- and loss-of-function lines for each class of *LRL* genes in *A. thaliana*. Angiosperm class I *LRL* genes are positive regulators of root hair development [11, 12]; *AtLRL1-2 AtLRL3-1* double mutants initiate root hairs that do not elongate (Figure 3B). Furthermore, longer root hairs develop in plants that constitutively overexpress *AtLRL1*, *AtLRL2*, or *AtLRL3* (Figures 3D–3F, 3I, and S4); plants transformed with *35S:AtLRL1* or *35S:AtLRL3* developed root hairs that were 899 ± 70 (mean \pm SD) μ m and 823 ± 27 μ m (Figure 3D, 3F, and 3I) and were longer than wild-type root hairs (474 ± 47 μ m; Figure 3A). Mutants with reduced activity of class II *LRL* genes—*AtLRL5-1^{T-DNA}* *35S:AtLRL4amiR-I* or *AtLRL5-1^{T-DNA}* *35S:AtLRL4amiR-II* plants—were indistinguishable from wild-type despite a significant decrease in steady-state levels of *AtLRL4* and *AtLRL5* mRNA levels (Figures 3C and S4). However, root hairs did not develop on wild-type

plants transformed with either *35S:AtLRL4* or *35S:AtLRL5* gene constructs demonstrating that *AtLRL4* and *AtLRL5* repress root hair elongation (Figures 3G, 3H, and 3I). We conclude that class I and class II *LRL* genes have antagonistic functions in the regulation of root hair development.

To test whether the class I and class II *LRL* genes reciprocally regulate each other's expression, we determined the steady-state levels of all *AtLRL* mRNAs in lines overexpressing each of the *LRL* genes. We could not detect changes of *AtLRL4* or *AtLRL5* steady-state mRNA levels in plants that overexpress *AtLRL1*, *AtLRL2*, or *AtLRL3* (Figure S4). By contrast, steady-state levels of *AtLRL3* mRNA were significantly reduced in *35S:AtLRL4* and *35S:AtLRL5* backgrounds (Figures 3J and S4). These data suggest that class II *LRL* genes repress the expression of the class I *AtLRL3* gene. We conclude that the two classes of *AtLRL* act antagonistically during root hair development and class II *LRL* genes have the potential to act as transcriptional repressors.

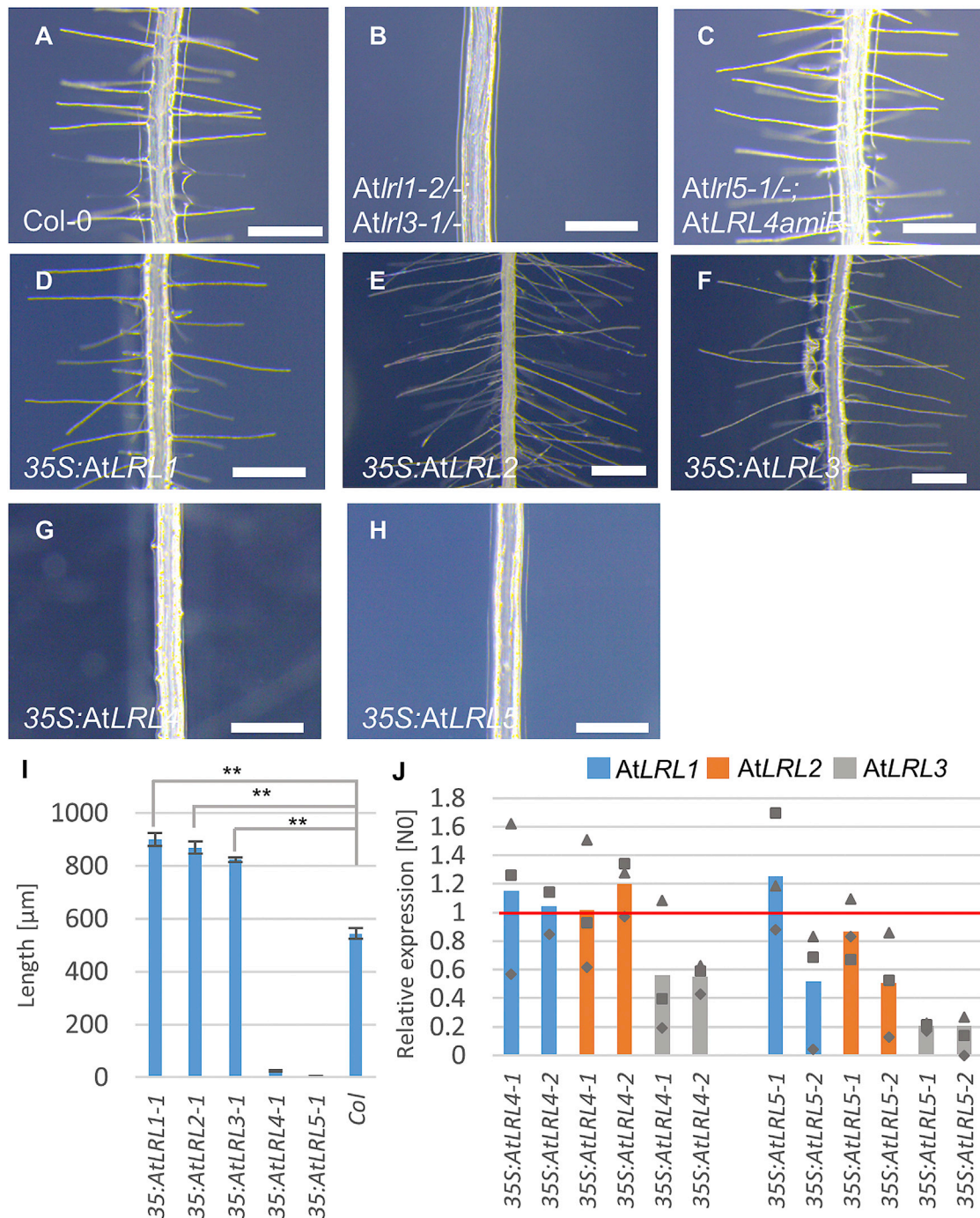


Figure 3. AtLRL Class I and Class II Act Antagonistically

(A–C) Root hair development in *AtLRL* loss-of-function mutants. (A) Wild-type root hair growth, (B) *AtLRL1-2; AtLRL3-1* double mutant (class I *AtLRL* mutant) with severe root hair growth defects, (C) *AtLRL5-1; 35S::AtLRL4amiR1* double mutant (class II *AtLRL* mutant), which is morphologically indistinguishable from wild-type. (D–H) Root hair development in *AtLRL* gain-of-function mutants. (D–F) Class I overexpression leads to increased root hair elongation. (D) *35S::AtLRL1*, (E) *35S::AtLRL2*, (F) *35S::AtLRL3*. (G and H) Class II overexpression leads to decreased root hair elongation. (G) *35S::AtLRL4* and (H) *35S::AtLRL5*.

(I) Quantification of root hair length in *LRL*-overexpressing lines. Student's *t* test *p* values <0.05 are marked with asterisks.

(J) qPCR analysis of class I *AtLRL* gene expression in two independent *35S::AtLRL4* and *35S::AtLRL5* transformants. Note the lower steady-state levels of *AtLRL3* mRNA in both overexpressing lines and decreased steady state of *AtLRL1* and *AtLRL2* mRNA in *AtLRL5*-overexpressing lines.

Scale bars, 500 μm . See also Figure S4.

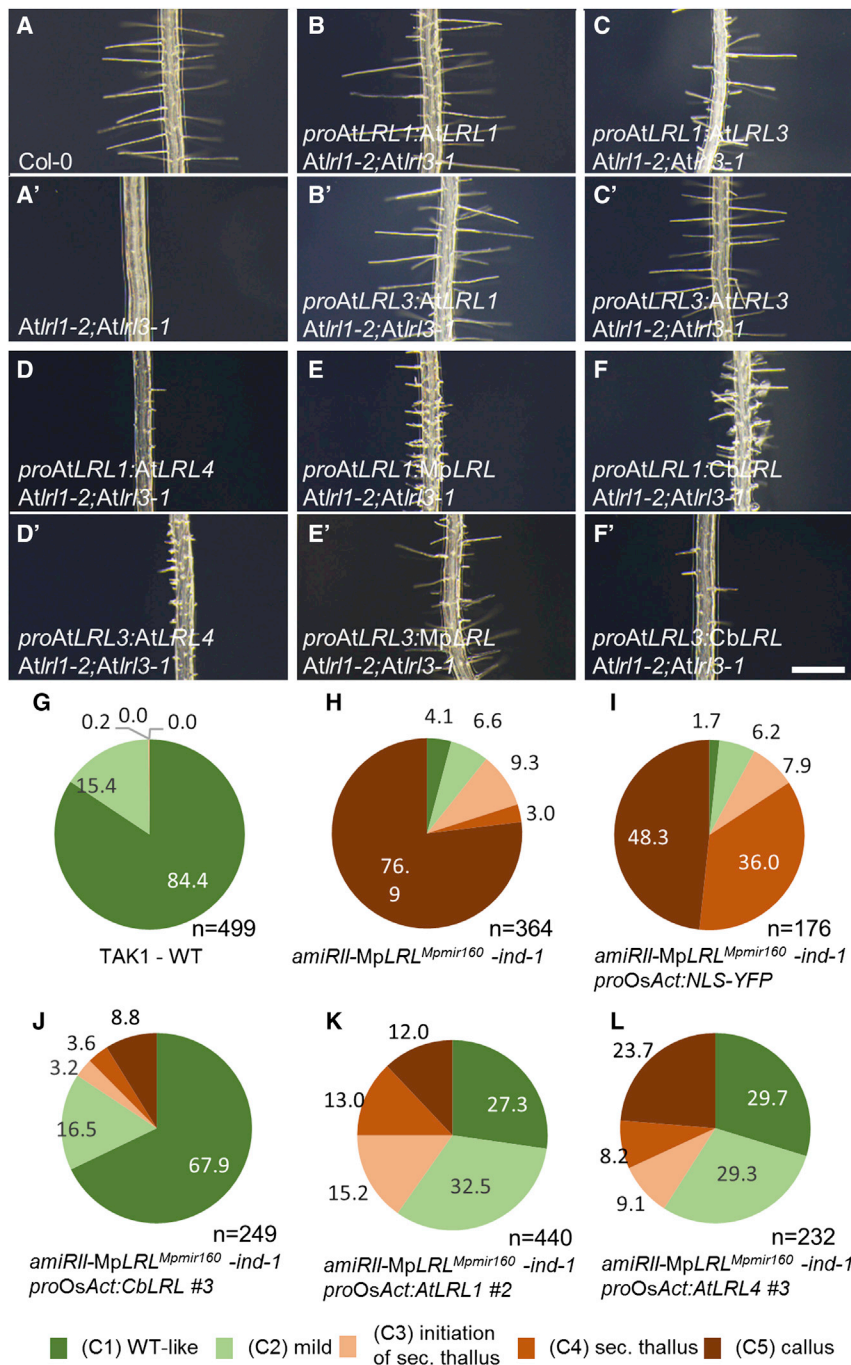


Figure 4. Conservation of LRL Gene Function among Streptophytes

(A and A') Root hair development in Col-0 wild-type (A) and the *AtLRL1-2;AtLRL3-1* double mutant (A'). Root hairs of the double mutant did not elongate. (B–C') Complementation of the *AtLRL1-2;AtLRL3-1* double mutant with a *AtLRL1* (B and B') or *AtLRL3* (C and C') cDNA, respectively, driven by either *proAtLRL1* (B and C) or *proAtLRL3* (B' and C'). Root hair growth was restored with all promoter-cDNA combinations.

(D and D') Complementation of the *AtLRL1-2;AtLRL3-1* double mutant with a *AtLRL4* cDNA driven by either *proAtLRL1* (D) or *proAtLRL3* (D'). Complementation of the root hair growth defect was not detected.

(E and E') Complementation of the *AtLRL1-2;AtLRL3-1* double-mutant phenotype using a *MpLRL* cDNA driven by either *proAtLRL1* (E) or *proAtLRL3* (E'). Partial restoration of root hair growth using either promoter.

(F and F') Complementation of the *AtLRL1-2;AtLRL3-1* double-mutant phenotype with a *CbLRL* cDNA driven by either *proAtLRL1* (F) or *proAtLRL3* (F'). Partial complementation could be detected using the *proAtLRL1* promoter.

(G and H) Distribution of phenotype classes of *M. polymorpha* plants grown for 2 weeks from gemmae in 20% EtOH vapor of wild-type (G) and *MpLRL^{Mpmir160}-ind-1* (H).

(I–L) Distribution of phenotype classes of *MpLRL^{Mpmir160}-ind-1* grown in 20% EtOH vapor and double-transformed with (I) *proOsAct:NLS-YFP*, (J) *proOsAct:CbLRL*, (K) *proOsAct:AtLRL1*, and (L) *proOsAct:AtLRL4*. Note the reduction of the strong class C5 phenotype in (J)–(L) compared to (I) and (H).

conditions (Figure 4H), the majority of gemmae in *amiRIL-MpLRL^{Mpmir160}-ind-1*; *proOsAct:CbLRL* plants were wild-type (67.9%; Figure 4J). This suggests that *CbLRL* can substitute for *MpLRL* function in *M. polymorpha*. In contrast, transformation of the *amiRIL-MpLRL^{Mpmir160}-ind-1* line with the *proOsAct:NLS-YFP* control construct did not restore wild-type development (1.7% developed wild-type phenotypes; Figure 4I). To determine whether relatively derived *LRL* genes can substitute for loss of *MpLRL* gene function, we expressed the *AtLRL1*

CbLRL Can Substitute for the Loss of LRL Gene Function in *M. polymorpha*

If *LRL* gene function was conserved after *C. braunii* and *M. polymorpha* diverged from a common ancestor, we predicted that expression of *CbLRL* would suppress the phenotypic defect caused by decreased *MpLRL* activity in *MpLRL* knockdown plants. We transformed the inducible *MpLRL* knockdown (*amiRIL-MpLRL^{Mpmir160}-ind-1*) plant with *CbLRL* cDNA under the control of a constitutive promoter. While defective gemmae developed on 92.2% of the *amiRIL-MpLRL^{Mpmir160}-ind-1* lines in inducing

(class I) and *AtLRL4* (class II) cDNAs using the *proOsACT* promoter in the background with decreased *MpLRL* activity. Only 27.3% and 29.7% of *proOsAct:AtLRL1*; *amiRIL-MpLRL^{Mpmir160}-ind-1* and *proOsAct:AtLRL4*; *amiRIL-MpLRL^{Mpmir160}-ind-1* lines developed wild-type gemma (Figures 4K and 4L). This indicates that the *A. thaliana* *LRL* genes cannot complement the defect in *MpLRL* loss-of-function lines as effectively as *CbLRL*. These data support the hypothesis that angiosperm *LRL* genes have functionally diverged since *M. polymorpha* and *A. thaliana* last shared a common ancestor.

AtLRL Class I and Class II Genes Have Diverged Functions

We tested the ability of CbLRL and MpLRL to restore root hair development in the *Atlr1-2 Atlr3-1* mutant background. Wild-type root hair growth was restored when double mutants were transformed with *proAtLRL1:AtLRL1* and *proAtLRL3:AtLRL3* (Figures 4B–4C', positive controls). Only partial restoration of root hair growth was observed in *proAtLRL1:CbLRL*; *Atlr1-2 Atlr3-1* or *proAtLRL1:MpLRL*; *Atlr1-2 Atlr3-1* or *proAtLRL3:MpLRL*; *Atlr1-2 Atlr3-1* lines (Figures 4E–4F). This is consistent with the hypothesis that *A. thaliana* LRL proteins have functionally diverged from LRL proteins in early diverging plants, although some aspects of the ancestral function have been conserved. Since expression of *AtLRL4* negatively regulate root hair development, and both *AtLRL1* and *AtLRL3* positively regulate root hair development, we predicted that expression of *AtLRL4* in the *AtLRL1* or *AtLRL3* domains of the *Atlr1-2 Atlr3-1* double mutant would not restore growth. As predicted, expression of *proAtLRL1:AtLRL4* or *proAtLRL3:AtLRL4* in *Atlr1-2 Atlr3-1* double mutants did not restore root hair elongation (Figures 4D and 4D'). This supports the hypothesis that *AtLRL1* and *AtLRL3* are functionally diverged from *AtLRL4*.

Taken together, these data support the hypothesis that LRL proteins have diversified during the course of land plant evolution. A single LRL gene controls thallus and rhizoid development throughout the early diverging land plant *M. polymorpha*, a function that partially has been conserved since *C. braunii* and land plants diverged from a common ancestor. Gene duplication followed by functional diversification gave rise to two classes of antagonistically acting proteins expressed in distinct but overlapping domains in relatively derived plants such as *A. thaliana*. This established a novel negative regulatory element controlling root hair outgrowth. Antagonistic regulation is critical in gene regulatory networks facilitating negative feedback or incoherent feedforward regulation [17]. It is therefore conceivable that the diversification of LRL gene function was an innovation that contributed robustness to the network, critical for the integration of internal signals with changing environmental conditions in derived groups of land plants.

ACCESSION NUMBERS

The accession numbers for the CbLRL and MpLRL sequences reported in this paper are GenBank: KX037431 and KX037432, respectively. The accession numbers for the *A. thaliana* LRL genes reported in this paper are TAIR: *AtLRL1* At2g24260, *AtLRL2* At4g30980, *AtLRL3* At5g58010, *AtLRL4* At1g03040, and *AtLRL5* At4g02590.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one dataset and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2016.04.060>.

AUTHOR CONTRIBUTIONS

Conceptualization, H.B. and L.D.; Methodology, H.B.; Investigation, H.B., A.T., and S.S.; Resources, H.S. and T.N.; Writing – Original Draft, H.B. and L.D.; Writing – Review & Editing, H.B. and L.D.; Funding Acquisition, L.D.; Supervision, L.D.

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