Growth regulation in tip-growing cells that develop on the epidermis

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Summary

Plants develop tip-growing extensions – root hairs and rhizoids – that initiate as swellings on the outer surface of individual epidermal cells. A conserved genetic mechanism controls the earliest stages in the initiation of these swellings. The same mechanism controls the formation of multicellular structures that develop from swellings on epidermal cells in early diverging land plants. Details of the molecular events that regulate the positioning of the swellings involve sterols and phosphatidylinositol phosphates. The final length of root hairs is determined by the intensity of a pulse of transcription factor synthesis. Genes encoding similar transcription factors control root hair development in cereals and are potential targets for crop improvement.

Introduction

Tip growth is a mechanism of cell expansion in which growth is restricted to a small area at the apex of the elongating cell (Figure 1) [1]. Virtually all land plants develop tip-growing cells, which may have roles in reproduction (pollen tubes), or function in nutrient uptake and anchorage (root hairs and rhizoids). Here we review recent discoveries, which reveal that an ancient mechanism controls the emergence of tip-growing cells from single epidermal cells, shed light on the molecular mechanisms that define the site of root hair outgrowth and demonstrate how the size of tip-growing root hairs is determined.

A conserved mechanism controls the initiation of epidermal cell outgrowth from the plane of the epidermis in land plants

Root hairs protrude from cells in the outermost layer of the root – the epidermis. Root hair development begins with outward swelling of the cell wall on the outer surface of the epidermis as a result of the activity of cell wall modifying enzymes, such as expansins [1-3]. At first the protuberance expands relatively slowly, but after the tip growth apparatus is in place, the growth rate accelerates to 1-2 $\mu\text{m}.\text{min}^{-1}$ [4].

A group of basic helix-loop-helix transcription factors – the class I ROOT HAIR DEFECTIVE SIX-LIKE (RSL) proteins – control the earliest stages of root hair development where the epidermal cell wall expands out of the surface plane. In *Arabidopsis*, two closely related class I *RSL* family genes *RHD6* and *RSL1* are expressed specifically in root epidermal cells that will develop root hairs, and mutants defective in *RHD6* and *RSL1* function are roothairless (Figure 2) [5-7]. Class I *RSL* genes were also recently shown to control root hair development in grasses, where they are sufficient for the development of root hairs when expressed in root epidermal cells. Overexpression of class I *RSL* genes in rice (*Oryza sativa*) or the grass model species *Brachypodium distachyon* resulted in the development of root hairs in every root epidermal cell [8,9]. These findings indicate that the angiosperm class I *RSL* genes are required for root hair cell differentiation and root hair outgrowth.

What was the ancestral role of the class I *RSL* genes? Microfossil evidence suggests that liverworts are likely the most ancestral lineage of land plants that diverged from the lineage leading to vascular plants soon after the first plants colonised land around 470 million years ago [10,11]. However, the relative phylogenetic positions of the early diverging groups of extant land plants – liverworts, mosses and hornworts – remain largely unresolved in recent molecular phylogenies. Therefore, characterising class I *RSL* gene function in more than one of these taxa can provide more reliable information on the ancestral role of the class I *RSL* genes.

The single copy class I *RSL* gene *MpRSL1* in the liverwort *Marchantia polymorpha* is required for the formation of filamentous rooting cells called rhizoids, which are morphologically similar to root hairs of vascular plants and form the entire rooting system in haploid liverwort gametophytes [6]. Similarly, the two class I *RSL* genes *PpRSL1* and *PpRSL2* in the moss *Physcomitrella patens* are required for rhizoid development [7]. In addition, the *M. polymorpha RSL1* gene is required for the development of a range of multicellular structures derived from single epidermal cells that bulge outward breaking the plane of the epidermis [6]. These structures include mucilage papillae, reproductive propagules called gemmae and multicellular slime papillae. Likewise, the development of bicellular papillae derived from single epidermal cells in *P. patens* is defective in the *PpRSL1 PpRSL2* mutants. These findings indicate that class I *RSL* genes of liverworts and mosses control the development of both unicellular and multicellular structures that develop from single epidermal cells. Together, these data suggest that class I *RSL* genes are likely to have functioned as positive regulators of the early stage of epidermal cell outgrowth during the morphogenesis of both unicellular and multicellular structures in the last common ancestor of land plants.

Evolution of antagonistic regulators of root hair elongation

LOTUS JAPONICUS Roothairless1-LIKE (*LRL*) family genes encode for basic helix-loop-helix transcription factors that are required for root hair development in the angiosperms *Lotus japonicus* and *A. thaliana* [12,13]. However, unlike class I *RSL* genes, they are also involved in other aspects of development and are expressed

throughout the plant [13,14]. The liverwort *M. polymorpha* has a single *LRL* gene *MpLRL*, which positively regulates plant growth and rhizoid development [14]. Likewise, *P. patens* mutants that lack the function of the two *LRL* genes (*PpLRL1* and *PpLRL2*) are stunted and do not develop rhizoids [15]. These data indicate that *LRL* genes function as general growth promoters and positive regulators of rhizoid development in early diverging land plants. *A. thaliana* has six *LRL* genes that belong to two subfamilies [14]. *LRL* genes from subfamily I are required for root hair elongation. In contrast, *LRL* subfamily II genes repress root hair development; plants overexpressing members of subfamily II develop short root hairs. Together these data suggest that an ancestral *LRL* gene promoted growth, and that during the course of land plant evolution, gene duplication led to the evolution of *LRL* subfamily I genes that promote root hair growth, and subfamily II genes that repress growth. The precise mechanism by which the genes in *LRL* subfamilies I and II respectively promote and repress growth remains to be defined.

Phosphatidylinositol phosphates and sterols mark the site of root hair outgrowth

The formation of the *RSL* gene-regulated cell surface outgrowth is accompanied by a number of changes that have recently been characterised in detail in *A. thaliana* root hair development. The plant hormone auxin is involved in positioning the site root hair outgrowth on the hair forming epidermal cells (trichoblasts). In wild type *A. thaliana*, auxin synthesised at the root tip is transported through the non-hair forming epidermal cell files towards the shoot by auxin transporter proteins, resulting in a tip-high auxin gradient along the root axis [16,17]. The non-hair forming cell files supply auxin to the adjacent root hair forming cell files, wherein the root hair initiation sites become established at the rootward end of each cell, towards the auxin maxima at the root tip [5,16,18,19]. Loss-of-function mutations in the auxin influx carrier *AUXIN RESISTANT1* (*AUX1*) result in more shootward root hair initiation [18,19]. Similarly, gain-of-function mutation that disrupts auxin dependent degradation of the Aux/IAA transcriptional repressor protein *AUXIN RESISTANT2* (*AXR2*) results in more shootward root hair initiation [5,20,21]. These results indicate that both auxin transport and auxin responsive gene expression are required for correct positioning of root hair initiation site on root epidermal cells. Furthermore, mutants for the class I *RSL* gene *rhd6* develop few root hairs that are also shifted shootward [5]. The root hair number and positioning defects in the *rhd6* mutants can be rescued by auxin treatment [5]. These findings indicate that auxin controls positioning the site of root hair outgrowth downstream of class I *RSL* genes.

Before root hair initiation, proteins required for positioning of outgrowth site accumulate in the plasma membrane at the future site of outgrowth. Phosphatidylinositol-4-phosphate 5-kinase 3 (PIP5K3) and RHO-RELATED PROTEIN FROM PLANTS2 (ROP2) GTPases are required for outgrowth site determination as their over-expression leads to initiation of multiple hairs from a single hair forming epidermal cell [22-24]. Furthermore, ROP GTPases are required for focusing cell expansion at the apex of elongating root hairs: plants expressing constitutively active ROP2 or ROP6 develop swollen depolarised root hairs [24,25]. Stanislas *et al.*, 2015

[26] demonstrated that ROP2, ROP6, PIP5K3 as well as DYNAMIN RELATED PROTEIN (DRP) 1A and DRP2B involved in Clathrin-mediated endocytic vesicle trafficking [27,28] co-localise in a distinct sterol-enriched domain at the future root hair outgrowth site. Furthermore, at the onset of outgrowth, the AGC VIII kinase D6 PROTEIN KINASE (D6PK) was recruited into this sterol-enriched membrane domain in sterol- and phosphatidylinositol-4,5-bisphosphate- (PtdIns(4,5)P₂) dependent manner (Figure 2) [26]. Therefore, while the role of D6PK in root hair development has not yet been established, these findings suggest that sterols and phosphatidylinositol phosphates contribute to the recruitment of proteins to a distinct plasma membrane domain focused at the future site of root hair initiation.

Arabidopsis class II *RSL* gene *RSL4* modulates root hair length

After root hair tip growth has been initiated from the swelling on the outer surface of the epidermal cell, the root hair begins to elongate rapidly. In Arabidopsis, the duration of the tip growth phase, and therefore the final length of root hairs, is defined by the levels of the class II RSL basic helix-loop-helix transcription factor ROOT HAIR DEFECTIVE SIX-LIKE4 (*RSL4*) [29,30]. Class I *RSL* genes (*RHD6* and *RSL1*) positively regulate the transcription of *RSL4* mRNA, which accumulates in a pulse-like fashion in root hair forming cells at the root elongation zone (Figure 2) [29,30]. *RSL4*-GFP fusion protein can first be observed 2 hours before the emergence of root hair outgrowth. *RSL4*-GFP protein level peaks 4 hours later, and then gradually declines [30]. Root hair elongation continues as long as *RSL4* protein persists in the nuclei of the hair cells. The *RSL4* protein has a D-box motif characteristic of proteins that become degraded by the 26S proteasome. Introducing a mutation that disrupts the *RSL4* D-box motif increased the longevity of the protein, resulting in extended tip growth phase and longer root hairs [30]. Together, these results indicate that the length of Arabidopsis root hairs is defined by the duration of the presence of *RSL4* protein in the nuclei of hair cells.

Root hairs have been shown to be important for uptake of phosphate, which is often the limiting nutrient for plant growth [31-34]. Phosphorus ions are negatively charged and bind tightly to positively charged clay particles in soil, and are thus relatively unavailable to plants. Plants have evolved many physiological, morphological and biochemical adaptations in order to increase phosphorus uptake under low phosphate conditions (Reviewed in [35]). One of these adaptations is the regulation of root hair length [36,37]. Increase in root hair length in low phosphate conditions results in increased surface area of the rooting system and exploration of larger volume of the soil, which has been shown to be associated with increased phosphate uptake and higher biomass [31-34,38].

The increase in Arabidopsis root hair elongation in response to phosphate starvation is exerted through *RSL4* [29]. Datta *et al.*, 2015 [30] discovered that a greater pulse of *RSL4* protein synthesis occurs in plants grown in low phosphate medium than in replete phosphate. This increase of *RSL4* protein synthesis during root hair initiation resulted in longer persistence of the *RSL4* protein and longer root hairs [30]. Low

phosphate can induce the development of root hairs in the *rhb6 rsl1* double mutant background, but not in the *rhb6 rsl1 rsl4* triple mutant background, indicating that the function of *RSL4* is required for the low phosphate response of the root hairs [29]. Therefore, *RSL4* integrates endogenous signals transmitted by the class I *RSL* genes, and environmental factors such as low phosphate to modulate root hair length.

Both auxin and *RSL4* positively regulate root hair elongation in *A. thaliana*. The expression of *RSL4* is increased in roots treated with artificial auxin, indicating that at least part of auxin-regulated root hair elongation is dependent on *RSL4* activity [29]. To elucidate how *RSL4* promotes root hair elongation, Vijayakumar *et al.*, 2016 [39] identified immediate downstream targets of *RSL4*. 34 putative *RSL4* downstream target genes were identified based on their increased expression after induction of *RSL4* activity in the presence of translational inhibitor. The putative *RSL4* downstream targets include genes that function in cell wall synthesis, membrane trafficking and cell signalling [39]. These genes are likely effectors of growth. Taken together, these data indicate that *RSL4* is a key regulator of root hair growth that integrates a variety of signals, such as low phosphate and auxin – and acts downstream of the class I *RSL* genes.

Root hair length in cereal crops is regulated by *RSL* genes

Longer root hairs allow the plant to explore and extract nutrients from a greater volume of soil. Therefore, engineering plants with longer root hairs may produce more nutrient uptake efficient crop varieties [34]. Class I and class II *RSL* genes are present in the genomes of cereal grasses [8,9,40]. Class I *RSL* genes in rice (*O. sativa*) and the cereal model species *B. distachyon* are required for root hair differentiation. Furthermore, expression of class I *RSL1* proteins in root epidermal cells is sufficient for root hair development in cereals; *O. sativa* or *B. distachyon* plants expressing class I *RSL* genes behind the strong constitutive rice ubiquitin promoter develop root hairs on every root epidermal cell [8,9]. These data demonstrate that *RSL* genes control root hair development in cereal grasses.

Recently, a pioneering study demonstrated how class II *RSL* genes play an important role in determining root hair length and cereal growth in low nutrient soils [40]. Bread wheat (*Triticum aestivum*) is an allohexaploid, with three subgenomes (AABBDD) that originated from two separate polyploidisation events between three diploid wild grass species. The first polyploidisation event produced allotetraploid durum wheat (AABB), which further hybridised with diploid goatgrass giving rise to allohexaploid bread wheat (AABBDD), the major wheat variety grown today [41]. Each of the three bread wheat subgenomes contains a copy of *TaRSL4*, a wheat homolog of the *A. thaliana* class II *RSL* gene *RSL4*. Therefore, each of these polyploidisation events was associated with an increase in *TaRSL4* gene copy number. Han *et al.*, 2015 [40] observed root hairs in natural and synthetic allotetraploid and allohexaploid wheat and their diploid progenitors to estimate the impact of the polyploidisation events that took place during wheat domestication on

root hair length. Root hairs of allotetraploid wheat (AABB) were significantly longer than those of their diploid ancestors [40]. These longer root hairs were correlated with higher steady-state *TaRSL4* mRNA levels, which is consistent with the hypothesis that RSL4 proteins control root hair length in cereals. Furthermore, overexpression of *TaRSL4* in an elite bread wheat cultivar resulted in longer root hairs and enhanced shoot biomass in plants grown under low nutrient conditions [40]. Therefore, the polyploidisation event that produced durum wheat (AABB) and the associated increase in class II *RSL* gene copy number is likely to have resulted in elevated class II *RSL* gene expression and therefore longer root hairs. It can be hypothesised that longer root hairs contributed to the suite of traits that increased vigor of the polyploid durum wheat relative to its diploid ancestors. Furthermore, these findings suggest that the role of class II *RSL* genes as modulators of root hair length is conserved between dicots and monocot cereal crops.

Conclusions

Recent discoveries have demonstrated how an ancient mechanism involving the *RSL* family of basic helix-loop-helix transcription factors control the formation of the polar outgrowth from epidermal cells at the plant surface. Research in *A. thaliana* has identified a suite of molecular events that occur at the site of outgrowth. Discovering the mechanistic link between these two processes and how regulatory mechanisms evolved during plant evolution will be a priority.

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