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Minireview

Recent Developments in the Cell and Molecular Biology of Root Hairs

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Recent results in root hair research show that these tip-growing cells are useful models in plant cell biology research. The review covers a range of topics, but there is particular emphasis on the use of mutants in molecular (genetic) analysis.

Key words: Cytoskeleton — Electrobiology — Freeze substitution — Hormones — Ion channels — Lectins — Mutants — Root hair — Tip growth — Vesicles

Root hairs are tubular-shaped tip-growing cells that arise from root epidermal cells called trichoblasts; they are thought to increase the absorptive capacity of the root by increasing the surface area (Clarkson 1985). Like all tip-growing cells, root hairs grow by the addition of cell wall precursors to the tip delivered by vesicles from the endomembrane system. Other examples of tip-growing cells are pollen tubes, fungal mycelia and moss and fern protonemata. Root hairs are non-dividing cells that elongate outside the body of the root, and their internal contents are easy to observe and experimentally manipulate.

Origin of root hairs

Most recent work has been done on *Arabidopsis*, in which the position of root hair cell files is determined by the underlying cortical cells, of which there are eight in *Arabidopsis*. Root hair cells are located over the longitudinal anticlinal wall (radial, i.e. perpendicular to the surface) between two underlying cortical cells (Dolan *et al.* 1994), and in young parts of the root, those epidermal cells over the longitudinal anticlinal wall (radial) wall are more cytoplasmically dense than neighbouring epidermal cells, thus distinguishing trichoblasts from non-hair-forming epidermal cells. Two other features distinguishing *Arabidopsis* epidermal cells (hair-forming and non-hair forming) are cell length, in which a file of shorter cells correlates with hair-forming cells, and deposits on the surface of non-hair-forming cells.

It is possible that a gene involved in hair cell fate has been found. Galway *et al.* (1994) have shown that the *TTG* gene (transparent testa glabrous) of *Arabidopsis* is required to specify epidermal cell fate and cell patterning in the root, because the normal positioning of root hairs over the anticlinal walls of cortical cells is absent. They suggest that the *TTG* gene normally provides, or responds to, positional signals, so that differentiating epidermal cells overlying cortical periclinal walls (i.e. epidermal cells directly overlying the cortical cells) develop a hairless state. Alterations in *TTG* activity caused developing epidermal cells to misinterpret their position and differentiate into inappropriate cell types. The *ttg* mutations are also associated with abnormalities in the morphology and organisation of cells within and surrounding the root apical meristem, and the gene may therefore have a fundamental role in development.

Cells in the meristem and elongation zone of the root are coupled symplastically through the plasmodesmata, but cells gradually become symplastically isolated from each other as they differentiate, by disconnection of the plasmodesmata. By the time hair outgrowth is visible the cells of the epidermis (including hair cells) are symplastically isolated (Duckett *et al.* 1994).

Regulators of root hair growth

Plant hormones may be regulators of root hair growth. Kinetin and abscisic acid (both at 10^{-9} to 10^{-5} M), brassinolide (10^{-10} to 10^{-6} M) and the synthetic hormone 2, 4-D (10^{-14} to 10^{-5} M) stimulate root hair elongation in clover, and marked promotion of growth occurs with application of 10^{-7} M 2, 4-D and 10^{-6} M kinetin (Izumo M., personal communication). In addition, kinetin and abscisic acid cause branching and other morphological changes in clover such as swelling of the tip or the base, twisting curling and bending (similar to the effects of rhizobia on clover). Swelling of the root hair base is also characteristic of some *Arabidopsis* mutants such as the *reb1-1* mutant isolated by Baskin *et al.* (1992) and the *rhd1* mutant isolated by Schiefelbein and Somerville (1990) and is also characteristic of changes in root hair development due to water stress (Schnall and Quatrano 1992). Hormones appear to have no effect on the position of the nucleus. The gibberellin inhibitor uniconazole (tested at 10^{-8} to 2.5×10^{-5} M) which is an inhibitor of gibberellin synthesis, strongly inhibits clover root hair growth, an inhibition that can be recovered by the application of exogenous gibberellin (Izumo M. and Katsumi M., personal communication).

Ethylene may be involved as a regulator of trichoblast differentiation in the epidermis (Dolan *et al.* 1994). In an

examination of the *ctr1* mutant phenotype of *Arabidopsis*, isolated by Kieber *et al.* (1993), which behaves as if it were grown in the presence of ethylene, Dolan *et al.* (1994) showed that these mutant plants have ectopic root hairs (i.e. located over the periclinal walls of the cortical cells as opposed to the normal position over the longitudinal anticlinal walls of the cortical cells).

Recently, Smith *et al.* (1994) have shown that the protein-phosphate inhibitors okadaic acid and calyculin-A (specific and potent inhibitors of the type-1 and type-2A families of serine/threonine protein phosphatases) block root hair growth (at concentrations as low as 3 nM) and alter the cortical cell shape of *Arabidopsis* roots. The results indicate that these protein phosphatases have a role in controlling the growth and development of roots and root hairs.

A study by Tretyn *et al.* (1991) on *Sinapis alba* root hairs demonstrated that auxins can act as external messengers in signal transduction.

Cytoplasmic streaming and the cytoskeleton

Advances in this area have slowed down in recent years. In an important earlier study on alfalfa root hairs development, Wood and Newcomb (1989) showed that there is a great variation in the speed of streaming, dependent on growth stage. Unfortunately Wood and Newcomb didn't report actual measurements of streaming speed.

The most recent comprehensive study on particle movement in root hairs (where particle movement is equated with cytoplasmic streaming) is by Ayling and Butler (1993) on tomato. Ayling and Butler recognised that it is difficult to distinguish experimentally-induced changes in particle velocity from those changes due to natural variability, and they used time-series analysis combined with piece-wise linear regression to provide an objective means of assessing experimental manipulation of streaming. Using data from auxin applications, Ayling and Butler were able to report that concentrations of auxin at or weaker than 10^{-7} M increases the velocity of streaming, and concentrations of 10^{-5} to 10^{-6} M significantly reduce streaming. Streaming rates vary between 0.6 and 0.9 μm per second in actively growing tomato hairs measured away from the tip. For clover, streaming during rapid early hair growth has been measured at an average of 3 μm (+/−0.87 SD) per second (Ridge, unpublished results).

More recently Shimmen *et al.* (1995) have shown that streaming in the root hair cells of *Hydrocharis* (which exhibits reverse fountain streaming) reaches 13 μm per second in transvacuolar strands. These authors also showed that the cessation of streaming caused by the toxin mycalolide B cannot be reversed (mycalolide B causes irreversible disorganisation of actin filaments) in comparison with the effects of cytochalasin B which are reversible.

There are many reports on the cytoskeleton in tip growing cells, but few for root hairs, and little recent work reported (for earlier references on immunfluorescence, freeze-substitution and dry cleaving work see Emons 1987, Ridge 1988, 1990a). Microfilaments have recently been proposed to have a 'channeling' function in the transport of coated vesicles after they bud from the plasma membrane during re-cycling of membrane at the tip (Ridge 1993a) and microtubules have been shown to be associated with micro-vesicles and plasma membrane-bound vesicle passages in *Vicia hirsuta* root hairs (Ridge 1995).

Vesicles

The tip-growing cell is a highly polarised cytoplasmic organisation geared to provide a dense population of secretory vesicles at the tip. There is little doubt that these vesicles contain pectic polysaccharides and cell wall precursors. For example, Sherrier and VandenBosch (1994) have shown that vesicles in *Vicia villosa* root hairs contain xyloglucan and methyl-esterified polygalacturonic acid, both cell wall matrix polysaccharides.

Vesicles are found mostly in the region between the nucleus and the tip, where the majority of the cell's cytoplasm is present, and they are concentrated in the apical dome of the tip where they exclude all organelles. Vesicles have also been found 'packaged' close to the plasma membrane, the package delineated by electron transparent outlines similar to the endoplasmic reticulum (Ridge 1993b, 1995). These outlines appeared to be continuous with nearby endoplasmic reticulum.

Vesicle sizes (after freeze-substitution) range from an average of 120 nm for secretory vesicles, a largest diameter of 95 nm for pyriform vesicles (a pear-shaped vesicle so far only reported for *Vicia hirsuta* by Ridge 1988) to 15 to 50 nm for micro-vesicles. Because of the report of the presence of micro-vesicles in root hairs (Ridge 1993b, 1995), it is suggested that vesicles 90 to 120 nm or larger be termed macro-vesicles. Emons (1987) reported secretory vesicles up to 300 nm in diameter for freeze-substituted *Equisetum hyemale* and *Limnobium stoloniferum*, and has seen small vesicles (A.M.C. Emons, personal communication) ranging between 25 and 60 nm in *Equisetum* and *Raphanus*, after freeze-substitution treatment.

Coated vesicles and pits have been suggested to be involved in plasma membrane turnover at the tip (Emons and Traas 1986, Ridge 1993a), and a close association of coated pits and vesicles with microfilaments has led to the proposal that microfilaments may guide the coated vesicles towards the endomembrane system (Ridge 1993a).

Electrobiology and gradients

There has been considerable recent interest in root hair membrane currents (Meharg *et al.* 1994), ion channel activity (Ehrhardt *et al.* 1992, Grabov and Bottger 1994, Gassmann and Schroeder 1994, Lew 1991, 1994, Ullrich and Novacky 1990, Lanfermeijer and Prins 1994, Felle *et al.* 1992), pH gradients (Gibbon and Kropf 1994, Felle 1994,

Brauer *et al.* 1995, Herrmann *et al.* 1995) and calcium gradients (Garrill *et al.* 1993, Schiefelbein *et al.* 1992, Ayling *et al.* 1994, Herrmann *et al.* 1995).

Growing root hairs of *Arabidopsis* have been shown to have two ion transport processes (Lew 1991, using micropipette techniques). One of these is a tetra ethyl ammonium-sensitive potassium ion current, and experiments showed that at resting potential there is a net inward potassium current. This potassium influx is suggested by Lew to be sufficient to 'drive' cellular expansion (or in this case tip growth) based on growth rates. Tetra ethyl ammonium also caused transient inhibition of tip growth. The other ion transport process in *Arabidopsis* root hairs is a plasma membrane proton pump, with a very high current density of 250 microamperes per sq cm, especially in young growing root hairs. This pump is presumed by Lew to drive the potassium influx required for cellular expansion (tip growth). Lew's technical system provides the possibility to study the physiology of root hairs in dynamic living state, a possibility agreed with by Meharg *et al.* (1994, using a two electrode method), who also showed the importance of correction for electrical wiring properties when studying membrane currents.

Gassman and Schroeder (1994, using patch clamp techniques) have shown that potassium uptake is regulated by two transport mechanisms in wheat root hairs (*Triticum aestivum*), but that the molecular mechanisms of potassium uptake are unknown. They conclude that these inward-rectifying channels can function both as physiologically important mechanisms for low affinity potassium uptake as well as regulators of membrane potential. Grabov and Bottger (1994) showed that Redox reactions are involved in the regulation of potassium channels in *Limnobium stoloniferum* root hairs.

Many compounds can be used to de-polarise the root hair membrane potential (see references above) but recently Ehrhardt *et al.* (1992) showed that Nod-factor (which is a lipo-oligosaccharide excreted by rhizobia as a signal molecule) is able to de-polarise the membrane of alfalfa root hairs (see also Kurkdjian 1995).

The role and distribution of calcium in root hairs is particularly important to study, because calcium ions are known to play a key role in many cellular processes in higher plants (e.g. cell division, cell expansion and cytoplasmic streaming). The role of calcium in tip-growing cells has been particularly well studied for pollen tubes (see e.g. Schnepf 1986, Heath 1990). With the goal of trying to understand the physiology of root hair development in relation to molecular-genetic experiments, Schiefelbein *et al.* (1992, using a calcium-specific vibrating probe) have shown that there is an extracellular calcium ion gradient at the tips of growing root hairs, but that no such external gradient is present at the sides of the hairs or at the tips of non-growing hairs. The Ca^{2+}-channel blocker nifedipine abolished this gradient (as well as tip growth). Schiefelbein *et al.* concluded that calcium ion influx through plasma membrane channels is required for normal root hair growth.

In a study on the effects of auxin on cytoplasmic streaming, Ayling *et al.* (1994) have showed that trans-plasma membrane electric potential is rapidly de-polarised by auxin, and that this is followed by a small and slow increase in cytosolic Ca^{2+}. Unfortunately Ayling *et al.* don't state whether they used growing or mature hairs for their study.

The regulation of cytosolic calcium has also been investigated in two studies for *Sinapis alba* root hairs (Felle *et al.* 1992, Felle 1994) in which they show that calcium ion homeostasis is regulated by a Ca^{2+}-ATPase in the plasma membrane. It is concluded by Felle's group that this ATP-ase is a major regulator of calcium ions during stress, particularly in those situations that shift cytosolic calcium steady state levels during processes of signal transduction. They also showed that Cl^- transport depends on the pH gradient across the plasma membrane rather than membrane potential. In a study on how the vacuolar-type H^+-ATPase regulates acidity within the vacuole, Brauer *et al.* (1995) have shown using the fluorescent pH indicator BCECF that the vacuolar pH of maize root hairs is an average of 5.8.

Cell wall (extracellular matrix)

Recent work on the localisation of cell wall polysaccharides has been made by Sherrier and VandenBosch (1994) on *Vicia villosa*, demonstrating the distribution of polygalacturonic acid and xyloglucan in the endomembrane system and cell wall. Their results show that de-esterified polygalacturonic acid is present on the external surface of the cell wall, but is not detectable within the cell, although chemical de-esterification revealed abundant antigen in Golgi bodies and secretory vesicles. Methyl-esterified polygalacturonic acid epitopes were detected within the medial and *trans* cisternae of Golgi bodies, secretory vesicles and throughout the cell wall, indicating that pectin is secreted in a neutral form and may be de-esterified *in muro* (in the wall). Xyloglucan was also detected within the *trans* cisternae, secretory vesicles and throughout the wall. Double labeling demonstrated that both polysaccharides occur simultaneously in the same Golgi bodies, and that secretory vesicles containing both polygalacturonic acid and xyloglucan deliver them to the cell wall at the growing tip.

One interesting aspect of the root hair wall is the presence of both lectins and lectin sugar haptens at the tip of root hairs. The surface binding of a lectin to root hairs was first demonstrated by Ridge and Rolfe (1986) who showed that the RCA-1 lectin (from *Ricinus communis*) not only bound to the tips of siratro (*Macroptilium atropurpureum*) root hairs but also to a specific area on each trichoblast surface, below which could be detected an aggregation of cytoplasm. Ridge and Rolfe suggested that this lectin binding detected certain cell surface sugars exocytosed at the bulge of initiation of the root hair, and is perhaps a sign of the initiation of the polarity that is required for tip growth. Diaz (1989) and Diaz *et al.* (1989a, b) showed that lectin was present on the root hair

tip surface of pea plants (presumably bound to a surface sugar), using an antibody raised against pea isolectin 2. From her work, it is now very convincing that lectins present at the root hair tips of peas are involved in aggregating and binding rhizobia to the surface of root hair tips (Diaz *et al.* 1989a, b). There are, unfortunately, no recent publications in this interesting area.

Molecular biology of development

The genetic analysis of root hair development has so far relied on the isolation of mutants, particularly of Arabidopsis (see e.g. Schiefelbein and Somerville 1990, Schiefelbein *et al.* 1993, Baskin *et al.* 1992). For root hairs, Aeschbacher *et al.* (1994) deduced that genetic analysis of root hair development is facilitated by the fact the root hairs are dispensable (Schiefelbein and Somerville 1990), agreed by Wen and Schnable (1994) working on *Zea mays*. Loci that have been identified by mutation fall into two major groups: those that affect the initiation of root hairs and those that are required for root hair elongation (Baskin *et al.* 1992, Schiefelbein and Somerville 1990).

The first sign of root hair emergence is a localised swelling of the trichoblast. For *Arabidopsis*, one gene that may play a role in this process is *RHD1* (root hair defective) (Schiefelbein and Somerville 1990, using ethyl methyl sulphonate as mutagen). The *rhd1* mutant forms an abnormal swelling or bulge on the trichoblast surface during initial hair formation. Other mutants also display abnormal epidermal swelling, such as the *Arabidopsis reb1-1* mutant isolated by Baskin *et al.* (1992), which may be a second gene that regulates the initial swelling process; and the *COBRA* gene (Benfey *et al.* 1993), which is a gene that affects general cell expansion, may also be involved.

Several loci involved in *Arabidopsis* root hair tip growth have been isolated, including *RHD2, RHD3, RHD4* (Schiefelbein and Somerville 1990) and *TIP1* (Schiefelbein *et al.* 1993). These genes may encode products that affect known tip growth factors, such as the cytoskeleton associated proteins, or calcium ion transport. The *rhd2* mutants possess "stubby" hairs that are apparently caused by an inability to expand past the initial swelling stage. The phenotypes *rhd3* and *rhd4* are "wavy hairs" and "bulging hairs" respectively, and have been interpreted as having defects in the control of cell expansion polarity at the root hair tip. By cross-breeding and producing double mutants, Schiefelbein and Somerville (1990) were able to show that the *RHD2* gene product is required before the *RHD3* or *RHD4* products, and that *RHD3* and *RHD4* products probably act in separate pathways. Although the *RHD3* and *RHD4* genes apparently affect root hairs specifically, the *RHD3* gene is required for normal cell expansion in many plant tissues, and has a similar action to the *COBRA* gene.

In consideration of the similar phenotypic response of hairs to exogenously applied plant hormones mentioned above, it will be interesting to see if the *REB1-1* and *RHD1* genes cause increases in levels of hormones such as kinetin and abscisic acid in the root hair or if the external application of such plant hormones causes a decrease in expression of these genes. In relation to this, the recent work of Masucci and Schiefelbein (1994) is highly significant. They have identified another *Arabidopsis* mutant, *rhd6*, that displays three defects: (a) a reduction in the number of root hairs, (b) an overall basal shift in the site of root hair emergence, and (c) a relatively high frequency of epidermal cells with multiple root hairs. All three phenotypes can be rescued by the inclusion of auxin or an ethylene precursor (1-aminocyclopropane-1-carboxylic acid) in the growth medium. The mutant phenotypes could be imitated by treating wild type *Arabidopsis* with an inhibitor of the ethylene pathway (aminoethoxyvinylglycine). These results indicate that the gene *RHD6* is possibly involved in directing the selection or assembly of the root hair initiation site through a process involving auxin and ethylene. Masucci and Schiefelbein also showed that the abscisic acid-resistant mutant *axr2* and the ethylene-resistant mutant *etr1* both had similar root hair phenotypes to *rhd6*, although less extreme. The *RHD6* gene is thus implicated in root hair initiation and is associated with the establishment of, or response to, root epidermal cell polarity.

The earlier work of Schnall and Quatrano (1992) has relevance in this discussion, in which they showed that abscisic acid elicits the "water-stress response" in *Arabidopsis* root hairs. This response causes root hairs to become short and bulbous, and although this is apparently similar to some of the mutants mentioned above, it is difficult to see from the micrograph in their paper. Nevertheless, according to the observations of Schnall and Quatrano, the *Arabidopsis* mutants *abi1* and *abi2*, which are insensitive to abscisic acid at the seedling stage, did not display the water stress response. Though the authors suggest only that abscisic acid may mediate the response of root hairs to water stress, it is easy to conclude that, in view of the work presented above, abscisic acid is involved in root hair development.

The *TIP1* gene appears to encode a product required for tip growth in both root hairs and pollen tubes (Schiefelbein *et al.* 1993), and *tip1* mutant plants produce short, branched root hairs, and slow growing pollen tubes.

As far as I can find in the literature, none of the *Arabidopsis* root hair genes have been cloned; however, with the development of tools to clone genes identified only by mutant phenotype, such as chromosome walking (using restriction fragment length polymorphism markers), analysis of root hair genes at the molecular level will undoubtedly soon be made.

Other *Arabidopsis* genes that were originally identified because the mutants display other phenotypes appear to be required for normal root hair growth. For example, the *dwf* and *axr2* mutants, which are auxin-resistant mutants, display defects in root hair formation. Cytokinin-resistant mutants (*ckr1*, Su and Howell 1992) produce shorter than normal root hairs. The phytochrome B mutants *hy3* produce longer hairs than normal.

Wen and Schnable (1994) have reported three genes (*RTH1, RTH2* and *RTH3*) that influence root hair development in *Zea mays*, and have suggested that root hairs are dispensable. The *rth1* and *rth3* mutants initiate normal root hair bulges (the authors call them primordia) that fail to elongate, and can thus be equated to the *Arabidopsis RHD2* gene isolated by Schiefelbein and Somerville (1990) mentioned above. One interesting difference between *rth1* and *rth3* mutants is that the *rth1* plants have pleiotropic nutrient deficiencies that affect growth, whereas the *rth3* mutants, with a similar root hair phenotype, grow vigorously, as do the *rth2* mutants, in which the root hair elongates only one fourth or one fifth of normal length. This forms the basis of Wen and Schnable's idea that root hairs are dispensable for normal growth under certain environmental conditions. It will be interesting to see what kind of homologies there are between the *Arabidopsis* and maize genes.

Molecular workers in the *Rhizobium*/legume field have shown transient interest in root hair genes (see Gloudemans and Bisseling 1989). The proteins of root hairs have been compared with total root protein preparations for soybean (Gloudemans *et al.* 1988), pea (Gloudemans *et al.* 1989), and clover (Gerhold *et al.* 1985) by one or two-dimensional gel electrophoresis. In addition Gloudemans *et al.* (1989) studied RNA populations of pea root hairs. In all these studies some ten root hair proteins or RNAs were identified that were not detectable in total root RNA or proteins, or were only present at much lower amounts. Rohm and Werner (1987) showed the occurrence of some root hair-specific proteins on the outer surface of the plasma membrane, using *in situ* labeling.

Gloudemans *et al.* (1988, 1989) using two dimensional gel electrophoresis isolated several pea mRNAs not detectable in total RNA preparations from roots. Most of these root hair-specific mRNAs occurred in elongating root hairs at higher levels than in mature hairs. They found that the expression of some genes in pea root hairs was affected by inoculation with *Rhizobium leguminosarum*. One gene, called *RH-42* was specifically induced, while the expression of another gene, *RH-44*, was markedly enhanced.

There is otherwise a paucity of work on the protein and molecular biology of root hairs. One of the reasons is the difficulty in isolating them from the rest of the root. However, Rohm and Werner (1987) have successfully developed a freeze 'fracture' technique whereby roots are dropped into liquid nitrogen and on stirring the roots sink to the bottom of the vessel but root hairs float and can thus be collected. Gloudemans *et al.* (1989) also successfully used this technique.

Concluding comments

Root hairs are in many respects ideal models for experimental plant cell biology. Their contents are easy to observe and record in real time using video microscopy with Nomarski optics, and they are particularly conducive to rapid-freeze, freeze-substitution techniques (Ridge 1988, 1990a, 1990b) (for the impact of freeze-substitution on biology, see Hippe-Sanwald 1993) which can be used as a preparation method for both fluorescence and electron microscopy.

There are, however, still many questions left to ask about root hair biology, especially about the mechanisms of tip growth. Apart from using the root hair to study such cell biological phenomena as streaming, calcium gradients, and through-flow within the endomembrane system, there are several special areas of interest. First, with the knowledge that there is more than one kind of vesicle found at the tip of root hairs, experimental work should be directed at discovering the role of these vesicles, in particular the pyriform vesicle, which has so far only been reported for a legume (and *Arabidopsis*, unpublished results). Second, there is clearly a cytoskeletal link between the tip of the hair and the migrating nucleus (Lloyd *et al.* 1987), and work should be directed at discovering the kinds of cytoskeletal-associated proteins or molecules that maintain this nuclear link with the growing tip. Third, and perhaps of greatest importance, there should be considerable encouragement to the isolation of more root hair mutants, not only for *Arabidopsis* but also for legumes, perhaps especially alfalfa because of its small size. We can learn the greatest amount by the isolation of mutants, because with modern techniques such as chromosome walking using RLFP markers, it will be possible to isolate root hair genes themselves.

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