Epidermal expression of a sterol biosynthesis gene regulates root growth by a non-cell autonomous mechanism in Arabidopsis

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Key words
Epidermal signalling; sterol biosynthesis; plant development; HYDRA1 gene; PIN proteins; sterol isomerase

Running title
Epidermal signalling requires sterol biosynthesis in Arabidopsis
Abstract

The epidermis has been hypothesized to play a signalling role during plant development. One class of mutants showing defects in signal transduction and radial patterning are those in sterol biosynthesis. The expectation is that sterol biosynthesis is a constitutive cell-autonomous process for the maintenance of basic cellular functions. The HYDRA1 (HYD1) gene of Arabidopsis encodes an essential sterol Δ8-Δ7 isomerase, and although hyd1 mutant seedlings are defective in radial patterning of several tissues, we show that the HYD1 gene is expressed primarily in the root epidermis. Cell type-specific transgenic activation of HYD1 transcription reveals that HYD1 expression in the epidermis of hyd1 null mutants is sufficient to rescue root patterning and growth. Unexpectedly, expression of HYD1 in the vascular tissues and root meristem, though not endodermis or pericycle, also leads to phenotypic rescue. Phenotypic rescue is associated with rescued patterning of the PIN1 and PIN2 auxin efflux carriers. The importance of the epidermis is in part due to its role as a site for tissue-specific sterol biosynthesis, and auxin is a candidate for a non-cell autonomous signal.
Introduction

A key question in plant development is how tissue patterning and cell expansion are coordinated in the course of organ growth. In the root of Arabidopsis, for example, the radial pattern is highly stereotyped, with predictable numbers of cells in each concentric layer of tissue, with coordination of cell expansion in each layer as the root grows (Dolan et al., 1993). This coordination of cell number and expansion is necessary, since plant cells are immobile and attached to each other. A failure of the coordination would likely to lead to growth and patterning defects.

The nature of such a coordination remains poorly understood. Mutant screens have led to the identification of genes essential for correct radial pattern in the Arabidopsis root, providing some insight into the molecular mechanisms involved. For example, the SCARECROW (SCR)/SHORTROOT (SHR) module controls ground tissue formation, and is characterized by the movement of the SHR protein from one cell layer (the stele), in which the gene is transcribed, to the cortex, where it regulates cell identity (Nakajima et al., 2001). The keule and knolle mutants exhibit radial defects such as bloated epidermal cells and very short roots, though the genes, that encode interacting components of the membrane trafficking system required for cytokinesis and cell wall construction, are expressed in all cells (Waizenegger et al., 2000; Assaad et al., 2001). Laser ablation experiments also highlight the importance of positional information in the regulation of root tissue patterning through as yet poorly defined signalling mechanisms (van den Berg et al., 1995, 1997). Non-autonomous signalling processes in radial patterning include the movement of transcription factors such as SHR between layers, and brassinosteroid (BR) signalling from the shoot epidermis has been implicated in regulating development of the ground and vascular tissues (Savaldi-Goldstein et al., 2007), and leaf shape (Reinhardt et al., 2007). The CRINKLY4 (CR4) receptor kinase of maize is expressed in the leaf epidermis and appears to signal to mesophyll cells, probably through an indirect mechanism (Jin et al., 2000; Becraft et al., 2001). In the root, epidermis-derived BR signalling also controls meristem size, through the modulation of, for example, the expression of the MADS-box transcription factor AGL42 in the quiescent centre (Hacham et al., 2011), though the transmitted signal remains unknown.

One class of mutants that exhibit radial patterning defects include the hydra1, jackel/hydra2 and sterol methyltransferase (smt) mutants, defective in sterol biosynthesis (Topping et al., 1997; Jang et al., 2000; Schrick et al., 2002, 2004; Souter et al., 2002). These are distinct from BR mutants, in that they cannot be rescued by exogenous supply of BRs (Topping et al., 1997; Schrick et al., 2000). Sterols are required for controlling membrane fluidity and permeability, and influence the activities of membrane-bound proteins (Grandmougin-Ferjani et al., 1997; Hartmann, 1998). Sterols are also implicated in the correct trafficking and localization of transporter proteins such as the PIN auxin efflux carriers (Willemsen et al., 2003; Men et al., 2008; Pan et al., 2009; Pullen et al., 2010),
and in cell plate construction (Peng et al., 2002; Schrick et al., 2004). Given the water-insolubility and presumed lack of mobility of sterols for thermodynamic reasons, the expectation is that they are synthesised in all or the majority of cells, permitting basic cellular functions; and would function in a cell-autonomous fashion. There is also a proposed role for HYD1 in miRNA function, with a requirement of ARGONAUTE1 (AGO1) activity being dependent on sterol-dependent membrane composition (Brodersen et al., 2012). Mutants such as hyd1, fk/hyd2 and smt1 exhibit significant patterning and growth defects in most cell types, and are typically seedling-lethal, suggesting essential roles in all cell types (Jang et al., 2000; Schrick et al., 2002; Souter et al., 2002; Willemsen et al., 2003).

To investigate cell autonomy of sterol action in Arabidopsis, we investigated HYD1 (At1g20050) expression and used transgenic activation systems to drive expression in different root cell types in the mutant background, to determine the cell types in which its expression is required for correct root development.

Results and Discussion

hyd1 seedlings exhibit abnormal morphogenesis and cell patterning and growth in the root (Fig. 1; Topping et al., 1997; Souter et al., 2002). The first evidence of defective radial pattern is seen during embryogenesis (Topping et al., 1997; Souter et al., 2002, 2004). Mutant seedlings typically develop multiple cotyledons of aberrant shape, a short hypocotyl and short root (Fig. 1B). The root has a defective apical meristem associated with aberrant patterning of surrounding cells (epidermis, columella, ground tissue, vascular tissue; Fig. 1C,D). Given the evident defects across several cell types, and the expectation that all cells contain sterols, we monitored spatial activity of the expression of a 2 kb fragment of the HYD1 gene promoter as a transcriptional fusion reporter with a β-glucuronidase (GUS, uidA) gene in transgens. The expression pattern in the primary root of transgens is shown in Fig. 1E. Unexpectedly, results show a spatially restricted expression pattern, localized to the lateral root cap, epidermis of root elongation zone and, less strongly, in the root differentiation zone (especially in trichoblast files), with some detectable expression in the root cortex. This is consistent with cell expression profiling visualised in the Toronto expression profiling browser tool (http://bar.utoronto.ca/eplant/; Winter et al., 2007) based on data from Brady et al., (2007). (Supplementary Fig. 1). These observations raise the question of how such a localized expression pattern leads to radial patterning defects across a wider range of tissues in the primary root of the hyd1 mutant.

While sterols are transported between intracellular membrane compartments via lipid transfer proteins (Saravanan et al., 2009), there is no evidence that they are transported between
cells. The question then is, how can localized expression of the HYD1 gene, which is essential for radial patterning throughout embryogenesis and post-embryonic growth, mediate the development of cell types in which it is not active? We have shown, for example, that vascular patterning is abnormal, and PIN1 localization is aberrant in the vascular cells, even though HYD1 is not expressed in those cells (Pullen et al., 2010). This suggests that a non-cell-autonomous signal is transferred from HYD1-expressing cells to non-expressing cells, to mediate wild-type tissue patterning.

To understand which cells might be sufficient and/or necessary for HYD1 expression to mediate processes essential for root growth and development, the full length HYD1 coding sequence (Topping et al., 1997) was cloned behind a variety of promoters and the UAS for use in mGAL4-VP16-GFP enhancer trap transactivation system (Laplaze et al., 2005), to drive HYD1 transcription in different root cell types: the columella and QC, epidermis, endodermis, pericycle and vascular cells. Promoter::HYD1 and UAS::HYD1 fusions were transformed into wild-type Arabidopsis, crossed with the hyd1 heterozygotes, and prospective homozygous mutant seedlings containing the promoter/UAS::HYD1 fusions were identified by genotyping and microscopy, for further analysis. Several independent transgenics were generated and typical expression patterns were identified in specific lines. For expression in the columella and QC we used the POLARIS (PLS) promoter (Casson et al., 2002) and the synthetic promoter DR5 (Sabatini et al., 1999), and the respective promoter-GUS expression patterns in both wild-type and hyd1 mutant root tips are shown in Fig. 2A-D. For epidermal and lateral root cap expression we used the GAL4 driver line J2551, shown in wild-type and hyd1 mutant roots in Fig. 2E-G; for endodermis, line J3611 (Fig. 2H-J); for pericycle line J0272 (Fig. 2K-M); and for vascular cells, line J0661 (Fig. 2N-P).

Expression in the hyd1 mutant reflects the aberrant tissue patterning, but both promoter::GUS and UAS::HYD1::GFP lines were identified that exhibited expression in the expected cell types. Examples are given in Fig. 2 of different seedling lines exhibiting the various UAS::HYD1::GFP expression patterns. These results provide the basis for the use of the promoter/UAS regulatory sequences to drive HYD1 transcription in specific cell types, to determine the effects on root development.

Genetically homozygous mutant seedlings expressing the HYD1 coding region in different cell types were grown for up to 21 days on vertical agar plates for phenotypic and growth analysis. While the mean length of wild-type roots was ca. 12.4 cm at 14 dpg, and for the hyd1 homozygous mutants was typically ca. less than 0.5 cm at 14 dpg, there were significant differences in the effects of expressing HYD1 in different cell types (Fig. 3A,B). The most significant restoration of primary root growth was in hyd1 seedlings expressing the HYD1 gene in the epidermis (i.e. J2551::HYD1; mean primary root length 9.1 cm at d 14), in the vascular tissues (i.e. J0661::HYD1; mean primary root length 3.2 cm at d 14) and in the root columella and lateral root cap (DR5::HYD1, PLS::HYD1; mean primary root length up to ca. 7.8 cm at d 14). Therefore in each of these lines, primary root length
was restored to ca. 60-80% that of wild-type by 14 dpg for epidermal expression, and with only ca.
30% wildtype growth seen with HYD1 expression in vascular tissue (Fig. 3A,C). 55-65% of wild-type
root growth was seen in seedlings with expression in the root columella and lateral root cap (Fig.
3B,D,E). Associated with significant restoration of root morphology and growth is an improved
patterning of cells in the root tip, seen as a regularized organization of the starch-containing
columella, though still with variable tiers of cells (Fig. 3F).

Root growth and cell patterning, including columella organization, has been linked to auxin
concentration and response in the root (Sabatini et al., 1999; Aida et al., 2004). Sterols have been
shown to be required for correct auxin-mediated gene expression (Souter et al., 2002, 2004) and for
PIN localization, including in the hyd1 and smt mutants (Carland et al., 2010; Pullen et al., 2010). PIN-
FORMED (PIN) proteins act as auxin efflux carriers, allowing auxin to be transported in a directional
manner to establish gradients across tissues, often with developmental or tropic (e.g. gravitropic)
consequences. Correct membrane sterol composition has been shown to be required for correct
PIN2 polarity and gravitropic response (Men et al., 2008). To determine whether the activation of
the HYD1 gene in specific cell types was associated with a restoration of PIN localization, PINs 1 and
2 were immunolocalized in both the mutant, wild-type, and transgenic lines expressing HYD1 under
control of cell type-specific promoters.

Results presented in Fig. 4 show that, as expected, the wild-type root tip shows PIN1
localized to the basal region of cells in the stele of the root, and PIN2 was localized to the apical side
of epidermal cells (Fig. 4A). In the hyd1 mutant, both abnormal cellular patterning and loss of polar
PIN1 and 2 localization are evident (Fig. 4B). Cellular patterning in seedlings expressing HYD1 in the
vascular tissues (J0661>>HYD1) (Fig. 4C) or pericycle (J0272>>HYD1) (Fig. 4D) is poorly restored, and
PIN expression and localization is variable, associated with relatively poor primary root growth (Fig.
3A). In seedlings expressing HYD1 in the epidermis (J2551>>HYD1), cellular patterning is similar to
wild-type, as is the localization of PIN2 and PIN1 (Fig. 4E). This is associated with relatively long
primary roots in these seedlings (Fig. 3A). In proPLS::HYD1 seedlings (Fig. 4F), radial patterning of
the root is restored close to wild-type, with an improvement of PIN localization compared to either the
hyd1 mutant or, for example, the vascular tissue line (J0661>>HYD1). The expression of two auxin-
regulated genes, IAA1 and IAA2, are known to be poorly expressed in the hyd mutants (Souter et al.,
2004) and activation on HYD1 in the epidermis (J2551>>HYD1), and also to some extent in the
vascular tissues (J0661>>HYD1) and root tip (PLS::HYD1) leads to some recovery of the expression
levels of both genes (Fig. 4G).

The evidence presented here suggests that the epidermis plays an important role in
controlling growth through its role as a site for sterol biosynthesis, and this involves a non-
autonomous signalling pathway, for which auxin is a strong candidate. Previous evidence
demonstrated a non-autonomous role for the epidermis in BR synthesis, but the signal involved was
not identified. Our data suggest that at least one coordinating signal across tissues is auxin, and the role of sterols in this context is to mediate correct localization and function of the PIN proteins, which are responsible for directional auxin transport. Sterols are known to control PIN polarization, and the data presented show that sterols are likely functioning in a non-autonomous fashion by mediating auxin gradient establishment, which in turn controls patterning and growth, through e.g. activation of the PLT/WOX5 mechanism (Aida et al., 2004; Sarkar et al., 2007). Exogenous auxin does not rescue the hyd mutant phenotype, supporting the view that gradients of auxin rather than absolute levels are required for correct development; and the hyd1 mutant lacks PIN3 proteins (Souter et al., 2002), which in wild-type accumulate distal to the quiescent centre and distribute auxin both down into the columella and laterally towards the epidermis and cortex (Friml et al., 2002). hyd1 also shows defective PIN1 and PIN2 localization and auxin patterning, associated with defective cell patterning (Pullen et al., 2010). The epidermis appears critical as a site of sterol biosynthesis via HYD1 - the hyd1 mutants fail to accumulate key sterols (Souter et al., 2002), and expression of the HYD1 gene specifically in the epidermis significantly rescues root growth and patterning of cells in the root tip.

Interestingly, HYD1 expression in the root cap (both columella and lateral root cap cells) also leads to significant root growth rescue, presumably by promoting PIN activity there to ensure stem cell niche activity - columella patterning is rescued, as well as root growth. Expression in the pericycle, endodermis or vascular tissues, on the other hand, has limited effects on root growth (Fig. 3), pointing to a mechanism distinct to, for example, the role of gibberellins in the endodermis (Ubeda-Tomas et al., 2008, 2009). These results show that the role of the epidermis in regulating root growth can at least in part be explained by its role in non-autonomous auxin signalling via sterol biosynthesis.

Materials and Methods

Plant material

The hyd1 mutant was identified in an insertional mutagenesis screen as described previously (Topping et al., 1997; Souter et al., 2002). The full length HYD1 cDNA sequence was cloned into the vector pCIRCE, and fused to the promoters DR5 (Sabatini et al., 1999), PLS (Casson et al., 2002) or UAS (Laplaze et al., 2005). The constructs were introduced into Arabidopsis thaliana by floral dip transformation (Clough and Bent, 1998). Homozygous T2 lines containing the UAS:HYD1 were crossed with the GAL4 driver lines J2551 (epidermal and lateral root cap expression); J3611 (endodermis); J0272 (pericycle); and J0661 (vascular cells), kindly provided by Dr. Jim Haseloff (Cambridge University, UK). Plants homozygous for all HYD1 constructs were crossed respectively
with plants heterozygous for the *hyd1* mutation, and selfed to identify progeny that was homozygous for both the original *hyd1* mutation and the *HYD1* fusion transgene, for further analysis. For growth assays, seeds were stratified, surface sterilized and grown on vertical agar plates containing half-strength Murashige and Skoog medium as described previously (Topping et al., 1997).

**Gene expression analysis**

RNA was extracted from seedlings, and gene expression measured by quantitative RT-PCR, with *ACTIN3* as an internal standard, as described previously (Rowe et al., 2016).

**Immunofluorescence microscopy and imaging**

*Arabidopsis* roots were fixed for 60 min at room temperature with 4% (w/v) paraformaldehyde in 0.1 M Pipes, pH 6.8, 5 mM EGTA, 2 mM MgCl₂, and 0.4% Triton X-100. The fixative was washed away with PBST buffer, and cells were treated for 8 min at room temperature with the solution of 2% (w/v) Driselase (Sigma) in 0.4 M mannitol, 5 mM EGTA, 15 mM MES, pH 5.0, 1 mM PMSF, 10 µg mL⁻¹ leupeptin and 10 µg mL⁻¹ pepstatin A. Thereafter roots were washed two times 10 min each in PBST and in 1% (w/v) BSA in PBST for 30 min, and incubated overnight with a primary antibody. The primary antibodies rabbit anti-PIN1 (1:150) and guinea pig anti-PIN2 (1:150). Specimens were then washed three times for 90 min in PBST and incubated overnight with goat anti-mouse TRITC and anti-rabbit FITC conjugated secondary antibodies diluted 1:200. After washing in the PBST buffer, specimens were mounted in the Vectashield (Vector Laboratories, Burlingame, CA) mounting medium. Images were acquired using Leica SP5 Laser Confocal Scanning Microscope using excitation at 488 nm line of Argon laser for FITC or 561 nm excitation of solid-state laser for TRITC. The emitted light was collected at 505-550 nm or 570-620 nm respectively.

Light micrographs were acquired using a Zeiss Axioskop microscope (Carl Zeiss Ltd, Herts, UK) equipped with Photometrics COOLSNAP™ cf colour digital camera (Roper Scientific Inc., Trenton, New Jersey, USA) and OpenLab3.1.1 software (Improvision, Coventry, UK). GFP signal in roots was imaged using Leica SP5 confocal microscope using 488 nm line of argon laser and emission was collected between 505 and 530 nm. The roots were mounted in double distilled water under a large (25x50 mm) zero-thickness coverslip.

**Competing interests**

No competing interests declared.
Acknowledgements

The authors are grateful to Prof. Klaus Palme (University of Freiburg) for providing antibodies against PIN1 and PIN2.

Author contributions

KL and JT conceived the project, ES, MP, GU, DL, N C-S, AS carried out experimental work, KL wrote the manuscript, JT, AS and PJH revised the manuscript.

Funding

Funded was received from the UK Biotechnology and Biological Sciences Research Council (grant number BB/C512210/1), awarded to KL.

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Figure Legends

Fig. 1. The hyd1 mutant and HYD1 expression. A. Wild type seedling, 6 dpg. Bar = 1 mm. B. hyd1 mutant seedling, 6 dpg. Bar = 1 mm. C. Wild type seedling root stained with lugol, 6 dpg. Bar = 100 µm. D. hyd1 seedling root stained with lugol, 6 dpg. Bar = 100 µm. E. proHYD1::GUS expression in wildtype seedling root at 7 dpg. Measurements in µm indicate the section distance from the root apex. Bar = 50 µm.

Fig. 2. Cell type expression of promoters in wild type and hyd1 mutants. A, B. proPLS::GUS expression in roots of wildtype (A) and hyd1 (B) seedling primary root tips at 7 dpg. C,D. DR5::GUS expression in roots of wildtype (C) and hyd1 (D) seedling primary root tips at 7 dpg. E-G. GFP expression in epidermal cells in GAL4 driver line J2551 in wildtype (E) and hyd1 (F,G) seedling roots at 7 dpg. H-J. GFP expression in endodermal cells in GAL4 driver line J3611 in wildtype (H) and hyd1 (I,J) seedling roots at 7 dpg. K-M. GFP expression in pericycle cells in GAL4 driver line J0272 in wildtype (K) and hyd1 (L,M) seedling roots at 7 dpg. N-P. GFP expression in vascular cells in GAL4 driver line J0661 in wildtype (N) and hyd1 (O,P) seedling roots at 7 dpg.

Fig. 3. Effect of cell-type specific expression of the HYD1 gene on root growth and columella organization. A. Primary root length of wildtype (WT), hyd1/WT heterozygous (HET), hyd1 homozygous (hyd1) and transgenic hyd1 seedlings expressing HYD1 in epidermis (epi), pericycle (peri), endodermis (endo) and vascular tissues (VT) at 7 and 14 dpg. B. Primary root length of wildtype (WT), hyd1 homozygous (hyd1) and transgenic hyd1 seedlings expressing HYD1 in root tips under the control of the PLS and DR5 promoters at 7 and 14 dpg. C. hyd1 mutant and transgenic hyd1 seedlings expressing HYD1 in the GAL4 driver line J2551 in epidermal cells at 7 dpg. Bar = 1 cm. D. hyd1 mutant and transgenic hyd1 seedlings expressing proPLS::HYD1 in root tips at 7 dpg. Bar = 1 cm. E. hyd1 mutant and transgenic hyd1 seedlings expressing DR5::HYD1 in root tips at 7 dpg. Bar = 1 cm. F. Root tips of wildtype (WT), hyd1 and transgenic hyd1 seedlings expressing HYD1 in the GAL4 driver line J2551 in epidermis (EPI), in line J0661 in vascular tissues (VT) and in root tips under the control of the DR5 and PLS promoters at 7 dpg.

Fig. 4. Effect of cell-type specific expression of the HYD1 gene on PIN proteins and auxin gene expression. PIN1 (red) and PIN2 (green) immunolocalization in wildtype root (A, A’), in hyd1 mutant root (B, B’), in hyd1 mutant root expressing HYD1 in the GAL4 driver line J0661 in vascular tissues (VT) (C, C’), in hyd1 mutant root expressing HYD1 in the GAL4 driver line J0272 in pericycle cells (D, D’) in hyd1 mutant root expressing HYD1 in the GAL4 driver line J2551 in epidermal cells (E, E’) and in hyd1 mutant root expressing HYD1 under the control of the PLS gene promoter (F, F’), at 7 dpg. G.
Expression of IAA1 (black bars) and IAA2 (open bars) in wildtype (WT), hyd1 mutant (HYD1), vascular tissues (VT), pericycle cells (Peri), epidermal cells (Epi) and root tip (PLS) relative to wildtype (value 1), determined by qRT-PCR. Means ± SD of 4 biological replicates.

Supplementary Fig. 1. HYDRA1 gene expression visualised in the Toronto expression profiling browser tool (http://bar.utoronto.ca/eplant/; Winter et al., 2007) based on data from Brady et al. (2007). Expression is shown in untreated roots, and in roots subjected to salt stress, iron deficiency and nitrogen application.
**A**

![Bar graph showing primary root length (cm) for different genotypes.](image)

**B**

![Bar graph showing primary root length (cm) for different genotypes.](image)

**C**

![Images of seedlings.](image)

**D**

![Images of seedlings.](image)

**E**

![Images of seedlings.](image)

**F**

![Images of seedling sections.](image)