

## The *HOBBIT* gene is required for formation of the root meristem in the *Arabidopsis* embryo

Viola Willemsen, Harald Wolkenfelt, Geert de Vrieze, Peter Weisbeek and Ben Scheres\*

Department of Molecular Cell Biology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands

\*Author for correspondence (e-mail: B.Scheres@biol.ruu.nl.)

Accepted 17 November 1997; published on WWW 13 January 1998

### SUMMARY

In *Arabidopsis*, the root meristem originates from the hypophyseal cell and from an adjoining cell tier that is distinct at the heart stage of embryogenesis. We have analysed mutations in the *HOBBIT* (*HBT*) gene that is essential for root meristem formation. *hbt* embryos display incorrect hypophyseal cell development from the quadrant stage onward. At the heart stage, the adjoining cell tier of *hbt* embryos develops abnormally, in that the activation of cell division and the formation of a lateral root cap layer are disturbed. Strong *hbt* mutants give rise to seedlings that lack an anatomically recognisable quiescent centre and

differentiated columella root cap cells, the cell types derived from the wild-type hypophysis. Furthermore, they have no mitotically active root meristem and lack a differentiated lateral root cap. Secondary roots of *hbt* mutants and roots obtained from cultured cells of *hbt* mutants have similar defects. Therefore the *HBT* gene is required for root meristem formation in different developmental contexts.

Key words: *Arabidopsis*, *HOBBIT*, Embryogenesis, Meristem, Pattern formation, Plant development

### INTRODUCTION

Embryogenesis in higher plants results in the formation of a juvenile plant, the seedling. The shoot meristem, cotyledon(s), hypocotyl, root, and root meristem are distinct pattern elements along the apical-basal axis of the seedling. The three main tissues, epidermis, ground and vascular tissue are arranged in radial layers, perpendicular to the apical-basal axis of the seedling. This seedling structure, with superimposition of apical-basal and radial pattern elements, is relatively uniform in higher plants (Natesh and Rau, 1984). The considerable variety in morphology of adult plant species is mainly brought about by the meristems at the opposite poles of the seedling, which produce the adult plant organs in the postembryonic phase of the life cycle (Steeves and Sussex, 1989).

In animals, embryogenesis can be divided into two steps. First, localised cues are deposited during axis formation to establish positional information (St Johnston and Nüsslein-Volhard, 1992). Subsequently, positional information is interpreted to establish regional identity (Ingham and Martinez Arias, 1992). In the brown alga *Fucus*, both sperm entry and environmental cues can orient the primary axis (Kropf, 1997). Cues involved in axis formation have not yet been identified in higher plants, but the *EMB30/GNOM* (*GN*) gene in *Arabidopsis* appears to be zygotically required for axis fixation (Mayer et al., 1993; Vroemen et al., 1996). Similarities of the GN protein to yeast proteins suggests a role in vesicle transport during axis stabilization (Laux and Jürgens, 1997; Busch et al.,

1996; Shevell et al., 1994). Directional vesicle transport is thought to drive asymmetric secretion of cell wall components during axis fixation in *Fucus* (Quatrano and Shaw, 1997).

The mechanisms underlying the interpretation of positional cues to establish regional identity in plant embryos remain to be elucidated. Early acquisition of identity along the apical-basal axis has been inferred from differences in cellular ultrastructure (Schulz and Jensen, 1968; Mansfield and Briarty, 1991) and from early region-specific gene expression (Lu et al., 1996). Genetic analysis has not yet uncovered genes involved in specifying the structurally distinct apical and basal cell, although *twin* mutants have been identified in which basal cell-derived suspensor cells are transformed to apical cell-like fates (Vernon and Meinke, 1994; Zhang and Sommerville, 1997). This demonstrates that the first embryonic cells have flexible fates. Mutants with broad deletions of seedling regions have led to the notion of early regional identity in *Arabidopsis* embryos. *gurke*, *fackel* and *monopteros* mutants are defective in the formation of the cotyledons/shoot meristem, the hypocotyl, and the hypocotyl/root, respectively (Mayer et al., 1991; Torres-Ruiz et al., 1996; Berleth and Jürgens, 1993). The earliest defects in these mutants suggest that the corresponding genes define genetically distinct apical, central and central/basal regions, respectively, in the preglobular embryo (Mayer et al., 1991; Jürgens, 1995; Fig. 1). These three regions, however, do not exactly correspond to the primordia of the cotyledon/shoot, hypocotyl, and root as seedling pattern elements (Fig. 1; Scheres et al., 1994). Therefore it has been

proposed that early regional specification is followed by cellular interactions that precisely define the different seedling elements (Jürgens, 1995). Furthermore, the apical defects in *gurke* embryos become obvious only after the globular stage of embryogenesis (Torres-Ruiz et al., 1996), and the *MONOPTEROS* gene may be primarily required for cell axialisation rather than for defining an embryonic region (Przemeck et al., 1996). Therefore, the stage at which zygotic genes are first required for regionalization is uncertain.

Here, we analyse mutations in the *HOBBIT* (*HBT*) gene that is essential for root meristem specification. We have traced back the *hbt* mutant phenotype to abnormal development of the basal embryo region from the quadrant stage onward. This region normally gives rise to the hypophysis, a founder cell for specific cell types in the seedling root meristem (Fig. 1, Dolan et al., 1993; Scheres et al., 1994). At later stages of embryo development, *hbt* mutants display aberrant development of those cells of the central region that adjoin the basal region, in that lateral root cap formation and the induction of cell division in the prospective root meristem are defective. These data suggest a role for the *HBT* gene product in early regional specification, and in later specification events that may involve cellular interactions.

## MATERIALS AND METHODS

### Plant growth conditions, plant strains and mutagenesis

For mutagenesis experiments the *Arabidopsis thaliana* ecotype Columbia-0 was used (containing a marker transgene consisting of the promoter of the vascular-expressed S-adenosyl-methionine-synthetase (SAM) gene fused to  $\beta$ -glucuronidase; Peleman et al., 1989). Dry seeds were mutagenised with freshly made 10 mM ethyl methane sulphonate (EMS) in water for 24 hours at 22°C. Seeds were sown on soil and grown in a plant chamber at 22°C, 75% humidity with a 16 hours light and 8 hours dark cycle. Single siliques representing 10,000 M<sub>1</sub> families were harvested, then seeds were suspended in 0.1% agarose and plated on a medium containing 0.8% plant agar (Duchefa) and 50  $\mu$ g/ml ampicillin. M<sub>2</sub> seedlings pooled in individual families were screened under a Zeiss stemi SV-6 dissecting microscope for root meristem defective mutants by pre-selecting mutants with severely reduced root length, and subsequent analysis of root cap structure in cleared specimens (see below). The M<sub>3</sub> progeny from siblings of candidate mutants with aberrant root cap structure was re-tested for mutant phenotype, and cleared ovule preparations (see below) were examined using Nomarski optics to detect abnormalities in embryo development. Six *hbt* alleles, 2311, 5721, 5859, 8052, 9620 and 9624 were recovered. 7.7% of the M<sub>1</sub> families segregated chlorophyll mutants, and 6 *monopteros* and 7 *gnom* alleles were recovered (complementation analysis by T. Berleth and U. Mayer, data not shown). The *hbt*<sup>611</sup> allele was recovered from a separate mutagenesis experiment performed as above but using Landsberg *erecta* seeds. The *hbt* alleles *GVI-20/1*, *GVII-24/1*, *G221-30/2* (ecotype Landsberg *erecta*) were provided by G. Jürgens (University of Tübingen) and the *hbt*<sup>56</sup> allele (ecotype Columbia-0) was provided by C. Bellini and H. Höfte (INRA, Versailles). The columella marker line 35S::B2 (Benfey et al., 1990) and the lateral root cap marker line LRC244 (Malamy and Benfey, 1996) were provided by P. Benfey (New York University); the 'basal embryo' marker line *AtEM101* (Topping and Lindsey, 1997) was provided by K. Lindsey (University of Durham).

### Complementation analysis

In complementation tests we combined *hobbit* alleles (with the dominant SAM-GUS marker) as donors with the Ler or *hbt*<sup>56</sup> alleles as acceptor, allowing selection of successful cross-pollination events. Non-complementation was observed in combinations of the reference

allele *hbt*<sup>2311</sup> with *hbt*<sup>56</sup>, *hbt*<sup>GVI-20/1</sup>, *hbt*<sup>GVII-24/1</sup>, *hbt*<sup>G221-30/2</sup> and *hbt*<sup>611</sup>, in combinations of *hbt*<sup>5859</sup> with *hbt*<sup>56</sup> and *hbt*<sup>GVII-24/1</sup>, in combinations of *hbt*<sup>5721</sup> and *hbt*<sup>9624</sup> with *hbt*<sup>GVI-20/1</sup>, and in combinations of *hbt*<sup>8052</sup> and *hbt*<sup>9620</sup> with *hbt*<sup>GVII-24/1</sup>, thus placing all *hbt* alleles in a single complementation group.

### Recombination mapping

*hbt*<sup>GVI-20/1</sup> heterozygous plants (ecotype Ler) were used to pollinate Col-0 plants and F<sub>1</sub> plants segregating mutants were selected. Individual F<sub>2</sub> *hbt* mutants were ground in liquid nitrogen, and suspended in 200  $\mu$ l ddH<sub>2</sub>O. 2–10  $\mu$ l of the DNA isolates were analysed using CAPS and micro satellite markers (Koorneef and Stam, 1992). Recombination frequencies in *n hbt* chromosomes were calculated as  $r = n_{Col}/(n_{Col} + n_{Ler})$ , and genetic distances were calculated using the Kosambi mapping function. Linkage was observed between *hbt* and the chromosome II markers m246 (8/52 recombinant chromosomes, 15.4 $\pm$ 5.5 cM) and GPA1 (24/294 recombinant chromosomes, 8.2 $\pm$ 1.6 cM).

### Phenotypic analysis

All *hbt* mutants were outcrossed prior to phenotypic analysis. For quantitative analysis, results of different sublines upon outcrossing were compared to assess effects of second-site modifiers. For the analysis of seedlings, seeds were stored at 4°C for 2 days and plated on medium containing 0.8% plantagar, and grown vertically. For embryo studies, siliques of soil-grown heterozygous plants were harvested and slit open under a dissecting microscope to collect ovules. Fixation, embedding, sectioning and microscopy for histological analysis of seedlings and embryos were performed as described previously (Scheres et al., 1994, 1995). For quantitative analysis of embryo phenotypes, ovules were cleared for 10 minutes in an 8:3:1 mixture of chloral hydrate: distilled water: glycerol (Mayer et al., 1991) and embryos were visualised using Nomarski optics on a Zeiss photomicroscope III.

The shoot apical meristem in mature embryos of *hbt*<sup>2311</sup>, *hbt*<sup>5859</sup>, *hbt*<sup>5721</sup>, *hbt*<sup>8052</sup>, *hbt*<sup>9620</sup>, *hbt*<sup>GVII-24/1</sup> and *hbt*<sup>G221-30/2</sup> was visualised by confocal laser scanning microscopy (CSLM) as described previously (Clark et al., 1995).

Cell numbers of the root were determined in chloral-hydrate-cleared seedlings by counting the cortical cells in files extending from the quiescent centre to the uppermost root hair. For root length measurements the seedlings were grown at 1/2 GM (8 g/l Duchefa plant agar, 2.2 g/l Murashige and Skoog salts incl. vitamins, 1% sucrose) for 10 days. After clearing of the seedlings the roots were measured from the tip to the uppermost root hair using a VIDAS RT image analysis system (Zeiss/Kontron) with a software package that is available on request (M. Terlou, Department of Image Processing and Design, Padualaan 8, 3584 CH Utrecht).

Starch granules in the columella root cap were visualised with 1% lugol solution (Merck) in 3-day-old seedlings grown on 1/2 GM. Seedlings were stained for 3 minutes, rinsed with water, cleared with chloral hydrate and photographed with Nomarski optics on a Zeiss photomicroscope III with a Agfa APX-25 film.

Cell numbers in the hypocotyl were determined in 10-day-old chloral hydrate cleared seedlings. Cortical cells were counted in files from the uppermost root hair to the cotyledon bifurcation point. Numbers of cells in the cotyledon epidermis of the mature embryo were counted in circumference and in median longitudinal sections showing both the cotyledons and the shoot apical meristem.

$\beta$ -glucuronidase activity in transgenic marker lines was visualised by staining for 2–16 hours at 37°C in 0.5 mg/ml X-gluc (Biosynth AG) dissolved in *n*-dimethyl-formamide, 0.1% Triton X-100, 0.5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>·H<sub>2</sub>O, 0.5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, and 50 mM sodium phosphate buffer, pH 7.2.

### Tissue culture

Tissue culture was performed essentially as described by Valvekens

et al. (1991). Seeds were sterilised in 5% sodium hypochlorite for 10 minutes, rinsed with distilled water and allowed to imbibe for 2 days at 4°C. The seeds were sown on 1/2 GM medium for 7 days. Mutant seedlings were fragmented and put on callus inducing medium (CIM) for 10 days. Calli were transferred to root or shoot inducing medium (RIM/SIM). Resulting roots were phenotypically examined as described above. Resulting shoots were removed and transferred to RIM (1/2 GM with 1 µg/ml indole butyric acid; IBA).

## RESULTS

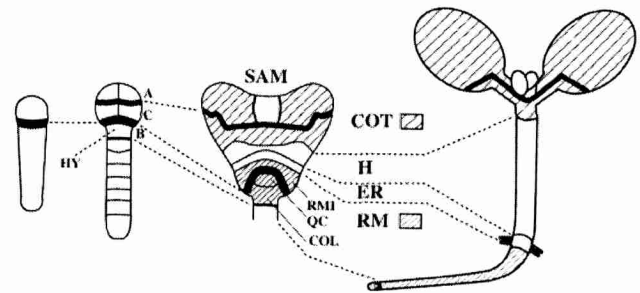
### Genetic analysis of root meristem defective mutants

The *Arabidopsis* root is formed from three adjoining cell groups in the heart stage embryo. The small 'embryonic root' at the junction of root and hypocotyl originates from the central region and its cells do not divide after embryogenesis (Fig. 1, ER). The root meristem, which is mitotically reactivated upon germination, originates from two other cell groups (Fig. 1, RM). 'Initial cells', which perform stem cell-like division patterns throughout development, originate from the cells of the central region that adjoin the basal region (Fig. 1, RMI). The quiescent centre and the columella root cap, including columella initials, originate from the hypophysis in the basal region (Fig. 1, QC, COL, HY). Columella initials add new layers to the columella root cap as the outer layers slough off.

To identify genes involved in the embryonic specification of the root meristem, we performed a three-step screening procedure. First, we preselected families that segregated mutants having the short embryonic root (Fig. 1, ER) but without significant root meristem activity. Second, these mutants were selected for anatomical defects in the normally very regular root cap. Third, remaining candidates were selected for aberrant embryonic development of the root primordium (see Materials and methods). Upon EMS mutagenesis of seeds and screening of 17,000 independent M<sub>2</sub> families using these criteria, seven families were identified that segregated seedlings with very similar stout appearance, which prompted us to name them '*hobbit* (*hbt*)' (Fig. 2). Four more families segregating similar seedlings, which originated from independent genetic screens (Jürgens et al., 1991; Desnos et al., 1996) were given to us.

Besides the root defect that was used as a selection criterion, *hbt* mutant seedlings have specific postembryonic developmental defects in the aerial parts that will not be discussed here, which lead to seedling lethality. Therefore genetic analyses were carried out using heterozygotes. All *hbt* families were subjected to complementation analysis as specified in Materials and methods, and no complementation was observed. Upon outcrossing to wild-type, all families displayed close to 25% segregation ratios when selfed as heterozygotes. We concluded that the *hbt* mutations are alleles of a single nuclear gene with no apparent gametophytic function. The *HBT* gene was mapped on the chromosome 2 interval between the markers m246 and GPA1 (see Materials and methods), which has been confirmed by RFLP fine mapping (data not shown).

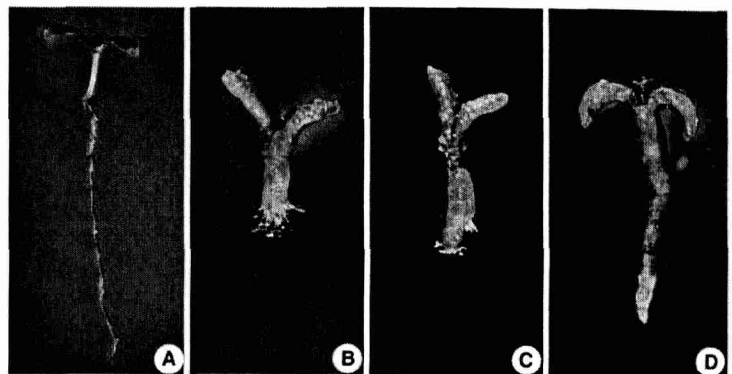
The frequency of mutant *hbt* alleles recovered in our Col-0 screen was similar to the frequency of



**Fig. 1.** Generation of apical-basal pattern elements during *Arabidopsis* embryogenesis. Left to right: 2-cell, octant, heart stage embryos and seedling. Thick lines: divisions separating apical (A), central (C) and basal (B) embryo regions (Jürgens, 1995). HY, hypophysis. Cell groups which give rise to seedling structures are indicated in the heart stage embryo. SAM, shoot apical meristem. COT, cotyledons; H, hypocotyl; ER, embryonic root; RM, root meristem; RMI, root meristem initials; QC, quiescent centre; COL, columella root cap.

*monopteros* and *gnom* alleles, and indicated that the screen approached saturation (see Materials and methods). In our screen, we identified several other mutants without a functional root meristem but with a distinct seedling appearance (Scheres et al., 1996). These additional mutants defined other complementation groups with mostly one allele per locus, which may suggest that they do not represent complete loss-of-function alleles.

Three classes of *hbt* alleles could be distinguished using root length as a phenotypic criterion (Fig. 2). We determined cell numbers in the root and established that root length correlates well with root meristem activity (Table 1). One class of Col-0 *hbt* alleles gave rise to seedlings with very short roots and strikingly similar appearance. This group was designated as 'strong' (Figs 2B, 3A; Table 1). The Col-0 *hbt<sup>es56</sup>* allele displayed residual root meristem activity and was designated as 'weak' (Figs 2D, 3B; Table 1). Trans-heterozygotes between the weak allele and strong alleles displayed a mean root length inbetween the mean values of mutants homozygous for the parental alleles (Fig. 3C). These observations collectively



**Fig. 2.** *hbt* seedling phenotype. Appearance of seedlings 7 days after germination on 0.8% plantagar. (A) Wild-type seedling. (B) *hbt<sup>2311</sup>* homozygote; (C) *hbt<sup>GVI-24/1</sup>* homozygote; (D) *hbt<sup>56</sup>* homozygote. Mutant seedlings are shown at 4× magnification of the wild-type seedling.

Table 1. Classification of the root phenotype of *hobbit* alleles

	WT	2311 Col-0	5721 Col-0	5859 Col-0	8052 Col-0	9620 Col-0	9624 Col-0	e56 Col-0	1611 Ler	GVI-20/1 Ler	GVII-24/1 Ler	G221-30/2 Ler
Root length (mm)*	μ 43.4§ σ 0.7 n 30	3.4 0.7 85	4.4 1.1 10	4.8 0.9 13	4.5 0.9 8	3.3 0.8 59	4.6 1.0 25	21.3 13.4 44	16.3 0.6 22	7.9 3.0 10	11.4 5.4 38	10.0 4.8 14
Cell number†	>>50	7.7	nd	10.2	8.2	9.6	8.7	>50	nd	nd	14.5	27.3
Lugol staining	++	–	–	–¶	–	–	–	++	+	+	+	+
Allele strength‡	–	s	s	s	s	s	s	w	**	**	**	**

\*Root measurements of 10-days-old seedlings grown on GM.  
†Cells in cortical files are counted in 10-days-old seedlings grown on GM.  
‡s= strong< w= weak (see Fig. 2).  
§Col-0 and Ler give the same results.  
¶3/33 seedlings had very few small black granules, different from granules found in wild type.  
\*\*Allele strength was not classified because of ecotypic specific modifiers (see text).

suggest that the strong alleles are nulls, and that *hbr<sup>e56</sup>* homozygotes contain residual *HBT* function. The difference in root size between the trans-heterozygotes and the *hbr<sup>e56</sup>* homozygotes indicates that the phenotype is remarkably sensitive to distinct residual levels of gene activity, which may signify that the *HBT* gene function influences root meristem activity in a quantitative manner.

All *hbt* alleles in the Ler background displayed residual root meristem activity at levels intermediate between strong and weak Col-0 alleles (Fig. 2C; Table 1). When the Ler alleles were crossed into the Col-0 background, *hbt* seedlings with a strong phenotype were frequently observed in the F<sub>2</sub> generation (171 of 204), implying that the difference in meristem activity is caused by a single ecotype-specific modifier and not by different levels of *HBT* gene activity. The high frequency of strong *hbt* mutants suggests that the linked *ERECTA* locus is not the modifier that is primarily responsible for the attenuation of the *hbt* phenotype.

The *HOBBIT* gene is required to specify hypophyseal cell derived cell types and lateral root cap

Our genetic screen was designed to identify genes that are specifically involved in root meristem specification, without primary functions in radial patterning of the embryo axis. Anatomical analysis reveals that the embryonic root and the hypocotyl of *hbt* mutants contain all differentiated cell types in a normal radial pattern, as depicted in Fig. 4. The inner tissue contains xylem and phloem elements in the normal diarch configuration and a pericycle layer, although this regular organisation becomes disrupted when multiple root primordia develop in the stele (Fig. 4B, arrow). Cortical and endodermal cell layers are present and the outer cell layer of the root region contains root hairs (Fig. 4). Cell morphology is less regular in *hbt* mutant seedlings but this trait is not apparent in the embryo (Fig. 8; see below), indicating that cellular patterning and cell divisions in the radial plane are not primarily affected.

We determined by microscopic analysis to what extent the organisation of the root meristem was affected in mutants carrying strong and weak alleles. As depicted in Fig. 5 the distal end of the root is specifically affected in the strong Col-0 mutants and in the Ler mutants. Irregular cell divisions are present in the columella and quiescent centre region, which provides a sharp contrast to the regularity observed in the wild type (Fig. 5A, QC, COL; Fig. 5B,C), and the typically layered structure of the lateral root cap is missing (compare Fig. 5A, LRC; Fig. 5B,C). In the weak *hbr<sup>e56</sup>* allele the anatomy is close to normal, although atypical divisions frequently occur in the QC, in the columella initials and in the cortical initials (Fig. 5D, QC, COL and C).

To investigate whether the abnormal anatomy of the distal root region was accompanied by cell differentiation defects, we tested whether root cap cells were properly differentiating in *hobbit* mutants. In wild-type roots, mature columella root cap cells contain characteristic starch granules (Fig. 5E; Table 1).

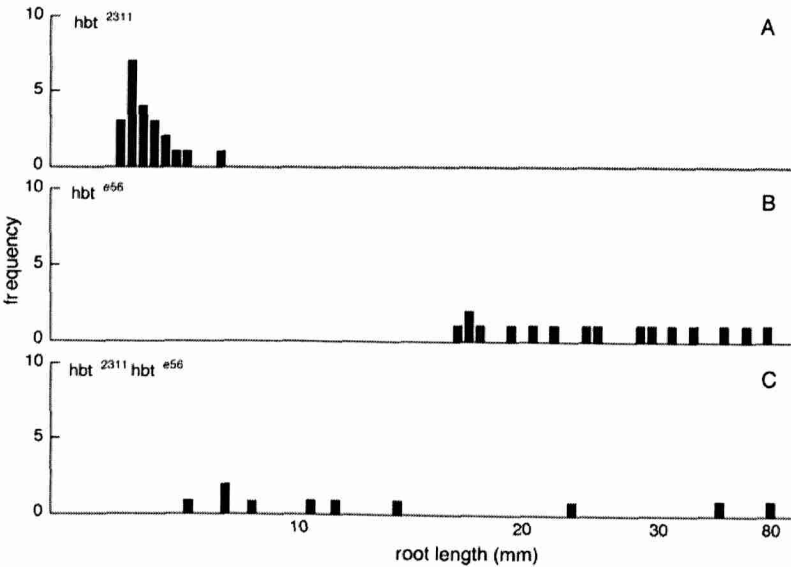


Fig. 3. Root length of strong and weak *hbt* alleles. Histograms depict the frequency distribution of root length plotted in a logarithmic scale. (A) Seedlings homozygous for the strong allele *hbt<sup>2311</sup>*; (B) seedlings homozygous for the weak allele *hbr<sup>e56</sup>*. (C) Transheterozygote *hbt<sup>2311</sup>/hbr<sup>e56</sup>* seedlings.



Table 2. Analysis of *hobbit* embryos

	2/4-cell†	Octant	Pre-globular	Globular	Early heart	Heart	Late heart	Torpedo	Mature	Total	Frequency (%)‡
2311	1/4*	7/38	6/24	6/66	9/27		7/39	15/79	11/40	62/317	19.5
5721	2/7	2/9	2/25	5/28	2/6					13/75	17.3
5859	0/1	0/7	7/28	4/21	6/38	1/2	13/44	3/27		34/168	20.2
9620	2/14	2/24	1/18	2/9	4/26					11/91	12.1
GVII-24/1	13/18	4/13	8/49	12/96	20/120		31/171	32/125	27/87	141/686	20.5
G221-30/2	3/8	2/8	1/28	5/27	12/36	4/28	10/53	11/51		48/238	20.2
Columbia-0	0/183	0/338	4/521	1/302	2/188	0/95	0/31	0/4		7/1662	0.4
Ler	4/8	1/25	2/60	1/18	0/19	2/57	0/32			10/219	4.5

Embryos with an aberrant pattern were counted in cleared ovule preparations from heterozygous parents.

\* (aberrant embryos/wild-type embryos)

† 2- and 4-cell embryo data were combined because Nomarski optics did not allow unambiguous staging.

‡ The frequency was calculated from the ratio of mutant and wild-type embryos at all stages.

Such granules were absent in the distal root cells of strong Col-0 *hbt* mutants (Fig. 5F; Table 1), strongly reduced in Ler *hbt* mutants (Fig. 5G; Table 1), and normally present in the weak allele *hbr<sup>es6</sup>* (Fig. 5H; Table 1).

To substantiate the notion of root cap specification defects in *hbt* seedlings, we analysed the expression of the markers 35S::B2 and PKU14, which show  $\beta$ -glucuronidase expression specifically in the columella root cap of wild-type seedlings (Fig. 6A,B). These markers were expressed in seedlings homozygous for the weak *hbr<sup>es6</sup>* allele (Fig. 6E,F) but not in homozygotes for the strong *hbt<sup>2311</sup>* allele (35S::B2,  $n=262$ ; PKU14,  $n=71$ ). This correlates with the presence/absence of starch granules.

We determined whether the down-regulation of gene expression in the columella root cap was tissue-specific by testing the *AtEM101* marker which, in the wild type, marks the basal embryo region and postembryonically the entire root meristem (Topping and Lindsey, 1997). This marker is expressed in seedlings homozygous for strong *hbt<sup>2311</sup>* alleles (Fig. 6G). Our data indicate that loss of *HBT* gene activity specifically interferes with all aspects of columella root cap specification, without disrupting the broader region-specific expression of *AtEM101*.

To test whether lateral root cap cells are specified, the lateral root cap marker LRC244 was tested (Fig. 6H; Malamy et al., 1997). LRC244 expression was absent in strong *hbt<sup>2311</sup>* mutants ( $n=40$ ) and present in homozygotes for the weak *hbr<sup>es6</sup>* allele (Fig. 6H). These results confirm the anatomical data on disruption of the lateral root cap layer in strong *hbt* mutants.

In conclusion, *hbt* mutants display not only specific defects in root meristem activity, but also specific defects in columella and lateral root cap identity. It is of note that the severity of all three defects is correlated in the allelic series (Table 1). Hence it is likely that these defects are directly caused by a decrease of the same activity of the *HBT* gene.

#### ***hobbit* mutants display aberrant development of the basal region at early stages of embryogenesis**

In order to trace the origin of the root meristem defect in *hbt* mutants, we compared *hbt* mutant embryos to wild-type embryos at different developmental stages. *Arabidopsis* embryogenesis has been described extensively (Mansfield and Briarty, 1991; Jürgens and Mayer, 1994), so relevant stages of wild-type embryo development will be described only briefly, followed by the differences observed in *hbt* embryos. Figs 7 and

8 depict wild-type embryos and embryos which are the progeny of plants heterozygous for *hbt* alleles. Mutant embryos with very similar phenotypes were observed for all Col-0 and Ler alleles in the expected frequency range of approximately 25% (Table 2), except those from parents carrying the weak allele *hbr<sup>es6</sup>*, where no apparent abnormal embryo phenotype was detected.

In quadrant stage wild-type embryos, the apical cell resulting from the first zygotic division has performed two longitudinal divisions, and the basal cell has divided horizontally (Fig. 7A, A and B). At the octant stage, the apical cell has formed the upper and the lower tier of the wild-type embryo proper (Fig. 7B, UT and LT, respectively). The basal cell derivatives form the extra-embryonic suspensor, except for the uppermost cell, the hypophyseal cell, which will be incorporated in the embryo proper (Fig. 7B, SU and HY). The first deviation from wild-type development that we observed in *hbt* mutant embryos was a vertical division in the hypophyseal progenitor cell occurring between quadrant and octant stage, instead of the normal horizontal division (Fig. 7E,F, arrowhead). Due to small numbers, we could not reliably determine the frequency of this first phenotype but we consider its occurrence in 3 Col-0 alleles (*hbr<sup>5721</sup>*, *hbr<sup>8052</sup>*, *hbr<sup>9620</sup>*) and its absence in non-*hbt*-segregating mutagenised Col-0 lines ( $n=183$ ; Table 2) as strong evidence for gene-specificity.

We occasionally observed aberrant cell divisions in the hypophyseal cell region of wild-type Ler embryos, indicating

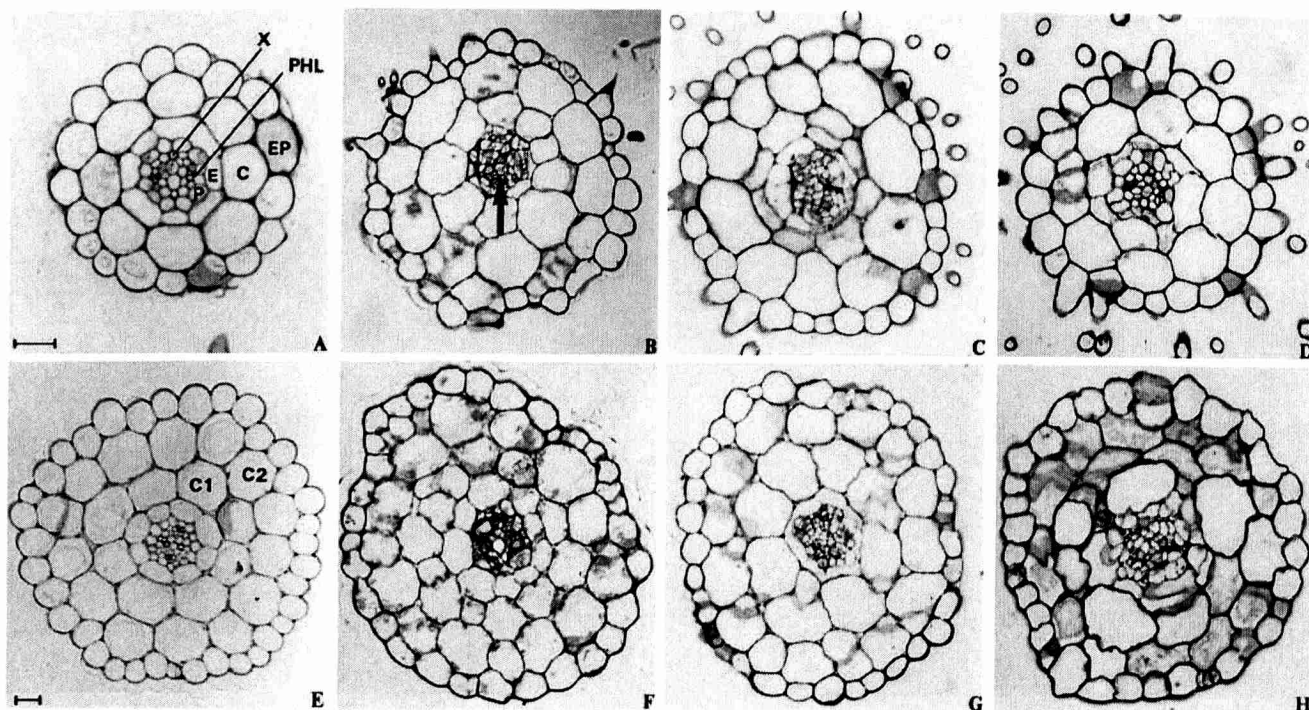
Table 3. Cell numbers of cotyledons and hypocotyls of *hobbit* mutants

	WT‡	2311 Col-0	8052 Col-0	9620 Col-0	GVII-24/1 Ler	G221-30/2 Ler
Epidermis cells in embryo	$\mu$ 52.8	52.9	nd	nd	50.1	nd
cotyledons*	$\sigma$ 6.1	5.8			3.8	
	$n$ 23	10			8	
Cortical cell number of seedling	$\mu$ 23.7	22.9	20.6	22.8	nd	21.0
hypocotyl†	$\sigma$ 1.9	3.5	3.9	2.4		2.2
	$n$ 14	15	11	15		2.1

\*Cotyledon epidermis cells of mature embryos were counted in circumference, in sections which were median for the shoot meristem and cotyledons.

†Hypocotyl cells do not divide postembryonically, so cells of cortical files were counted from the uppermost root hair to the junction of the vascular bundle of 10-day-old seedlings grown on 1/2 GM.

‡Col-0 and Ler give the same results.



**Fig. 4.** Mature root and hypocotyl regions of 7-day-old wild-type and mutant *hbt* seedlings. Toluidine blue stained transverse sections. (A-D) Root; (E-H) hypocotyl. (A,E) Wild-type; (B,F) *hbt*<sup>2311</sup>; (C,G) *hbt*<sup>GVII-24/1</sup>; (D,H) *hbt*<sup>e56</sup>. X, xylem (stains light blue); PHL, phloem P, pericycle; E, endodermis; C, cortex, EP, epidermis. In the hypocotyl two cortical layers are present (C1,C2). In B the arrow indicates the root primordium. Bar (A-D and E-H), 25  $\mu$ m.

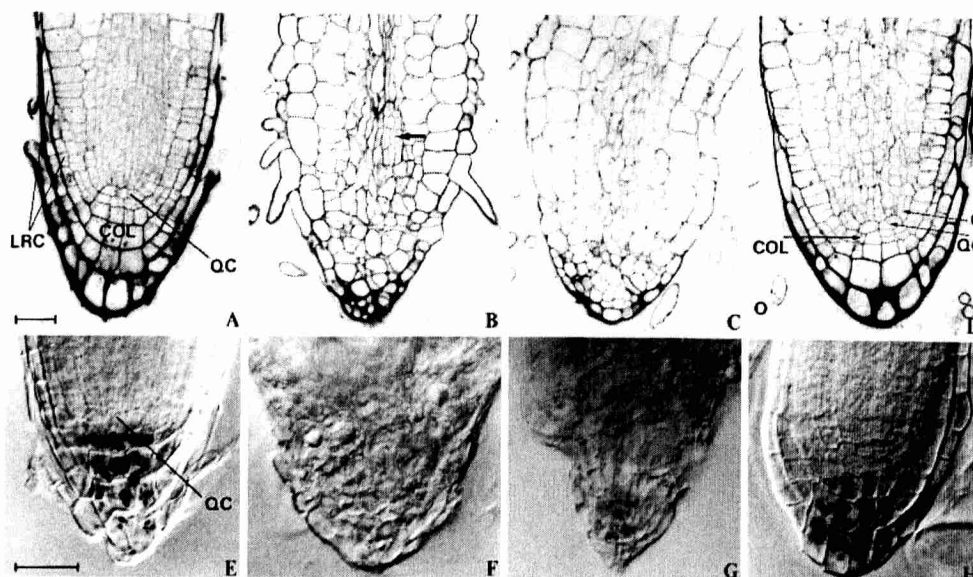
that the regulation of cell division at early stages is not as strict as in Col-0 embryos (Table 2, Col-0 and Ler). Therefore we did not draw conclusions from the earliest phenotypes observed in Ler *hbt* embryos. From globular stage onwards, the defects in the hypophyseal cell region of the Ler alleles become statistically significant (Table 2).

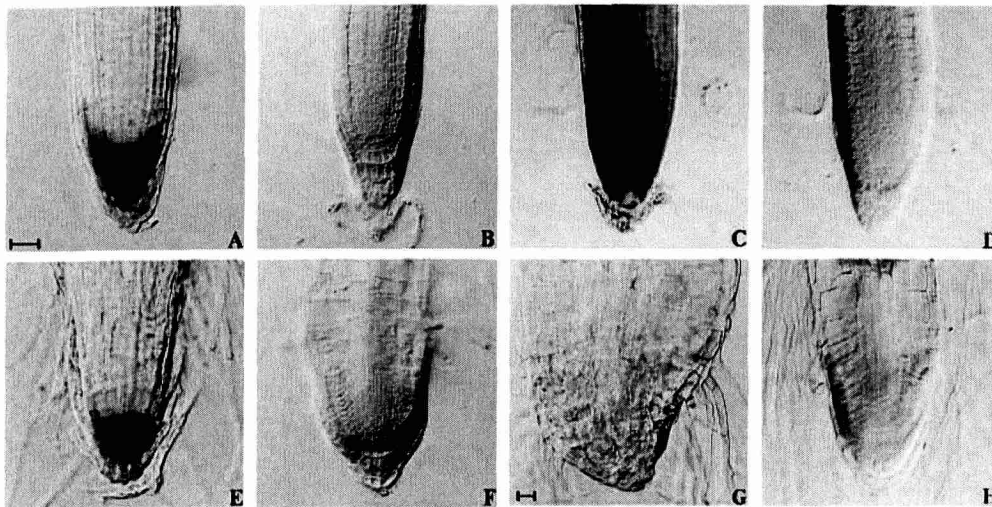
In wild-type late globular embryos, the hypophysis forms the

lens-shaped progenitor cell for the quiescent centre and the columella precursor cell (Fig. 7C,D, QC and COL). In *hbt* embryos the asymmetric division that creates the lens-shaped cell is either absent or accompanied by additional, atypical divisions (Fig. 7G,H). The atypical organisation of the hypophyseal cell region is the only anatomical defect in *hbt* embryos up to the heart stage of embryogenesis, it persists

**Fig. 5.** Anatomy and cell fate in the root tip of *hbt* mutants.

(A-D) Median longitudinal astra blue stained sections of 7-day-old seedlings; (E-H) whole-mount preparations of lugol-stained seedlings viewed by Nomarski optics to visualise starch granules (dark grains) in the columella. (A,E) Wild-type; (B,F) *hbt*<sup>2311</sup>; (C,G) *hbt*<sup>GVII-24/1</sup>; (D,H) *hbt*<sup>e56</sup>. QC, quiescent centre; COL, columella; LRC, lateral root cap. In B the arrow indicates the lateral root primordium. Bar, 25  $\mu$ m.





**Fig. 6.** Marker expression in wild-type and *hbt* mutant seedlings. (A-D) Wild type; (E,H) *hbt<sup>e56</sup>* homozygotes; (G) *hbt<sup>2311</sup>* homozygotes. (A,E) Root cap marker 35S::B2; (B,F) root cap marker PKU14; (C,G) root meristem marker AtEM101; (D,H) lateral root cap marker LRC244. Bar (A-F,G and H), 25  $\mu$ m.

throughout later stages (Fig. 7J,L) and it occurs at the expected frequency (Table 2).

In conclusion, *hbt* embryos show an early defect in the development of the hypophyseal cell region. This is consistent with the lack of a columella root cap and a recognisable quiescent centre (the cell types normally formed by the hypophysis) in seedlings homozygous for strong *hbt* alleles.

#### **hobbit mutants display aberrant meristem initial activity and lateral root cap development at late stages of embryogenesis**

At the heart stage of wild-type embryogenesis, the initials of the root meristem are first visible as separate cells that adjoin the hypophyseal cell region, and the first periclinal divisions for the formation of a lateral root cap layer take place (Fig. 8A, LRC). In torpedo-stage *hbt* embryos, periclinal divisions of the epidermal cells to form a lateral root cap are frequently skewed (Fig. 8F, arrow) or absent. The occasional presence of such periclinal divisions in strong *hbt* mutants indicates that the *HB*T gene influences their frequency but is not critical for their occurrence, as it is for the expression of the LRC244 lateral root cap marker (see previous section).

In mature wild-type embryos, the root meristem initials have produced a distal region of the root which is enclosed by the lateral root cap (Fig. 8E, dotted line). No properly formed lateral root cap is present in mature *hbt* embryos. Furthermore, the number of cell divisions in the root apex is reduced, which can be deduced from the anatomy of *hbt* seedlings as follows. In wild-type Col-0 roots, the average number of cortical cells in files between the hypocotyl boundary and the hypophyseal-cell-derived region prior to the onset of postembryonic cell division is 17 (Scheres et al., 1994; Cheng et al., 1995). In strong *hbt* mutants, this number is only 8-10 (Table 1), which equals the cell number of the wild-type 'embryonic root' that is not derived from the root meristem initials (Scheres et al., 1994). In addition, epidermal cells in the root region of strong *hbt* mutants are birefringent and carry root hairs in irregular positions (data not shown). Both these traits are characteristic for the embryonic root (Scheres et al., 1994). These observations indicate that the reduction of cell number in the

*hbt* root apex is due to minimal or no mitotic activity of cells flanking the hypophyseal cell region at the heart stage, which are the root meristem initials in wild type. Mitotically active root meristem cells and a lateral root cap are absent in strong *hbt* mutants. Collectively, both the embryonic and the seedling defects suggest that root meristem initials are not properly specified in *hbt* embryos.

The hypocotyl and cotyledons of the mature embryo contain all cell layers with the typical radial arrangement and cell shape found in wild-type embryos (Fig. 8G). Counting of the epidermal cells in circumference of the cotyledons revealed no difference between wild-type and *hbt* mutant embryos (Table 3). Cell numbers in cortical files of the seedling hypocotyl in wild type and *hbt* mutants also did not differ significantly (Table 3). These numbers reflect cell numbers in the embryo, as hypocotyl cells do not divide postembryonically. We concluded that *hbt* mutant embryos contain normal numbers of cells (with the exception of the root apex).

The postembryonic defects in *hbt* seedlings include abnormal development of the shoot meristem (data not shown), which raises the question of whether an aberrant shoot apical meristem is formed in *hbt* embryos. Microscopic sections of mature *hbt* embryos revealed a group of small cells that formed a bulge between the cotyledons as in wild type (Fig. 8I,J, arrowhead). To further address whether this cell group resembled a characteristic shoot meristem, we examined confocal sections of embryos stained with propidium iodide. This specifically detects a cluster of small cells between the cotyledons of mature wild-type embryos. Such a cluster of cells is notably absent in mutants with defective specification or maintenance of the shoot meristem (Endrizzi et al., 1996; Clark et al., 1996). In mature *hbt* embryos, such a cluster with the typical appearance of a wild-type shoot apical meristem is found (Fig. 8K,L, arrowhead). Hence we could not detect differences in embryonic specification of the shoot apical meristem between wild-type and *hbt* mature embryos, although our phenotypic criteria are less stringent than the ones applied to the root meristem.

Taken together, we deduce from the *hbt* embryo phenotype that the *HB*T gene is specifically required for the formation of



the root meristem during embryogenesis. First, it is required in the hypophyseal cell region at the quadrant/octant stage, when this cell is normally formed. Second, it is necessary for appropriate cell division and lateral root cap specification in the adjoining root meristem initials at the heart stage, when these cells become distinct in wild-type embryos.

### The *HOBBIT* gene is required for lateral and adventitious root formation

The primary root of higher plants is laid down during embryogenesis. After germination, lateral root primordia initiate from pericycle cells of the primary root (cf Malamy et al., 1997). Lateral roots develop an apical meristem that is similar in structure and function to the primary root meristem. Novel root primordia can also arise at other locations, e.g. from within the hypocotyl, to give rise to adventitious roots. In strong *hbt* mutants, stele cells in the root and hypocotyl divide inappropriately at later stages of seedling development. They give rise to clusters of cells resembling root primordia, which subsequently abort development (Figs 4, 5). These clusters never express the 35S:B2 root cap marker ( $n=21$  seedlings). In weak *hbt<sup>es56</sup>* mutant seedlings, lateral roots are formed but they display the mild defects typical for primary roots of *hbt<sup>es56</sup>* seedlings. In seedlings homozygous for the Ler *hbt* alleles, short lateral roots with similar defects as the primary root are frequently formed. Thus, *hbt* allele strength is comparable in both primary and secondary root formation.

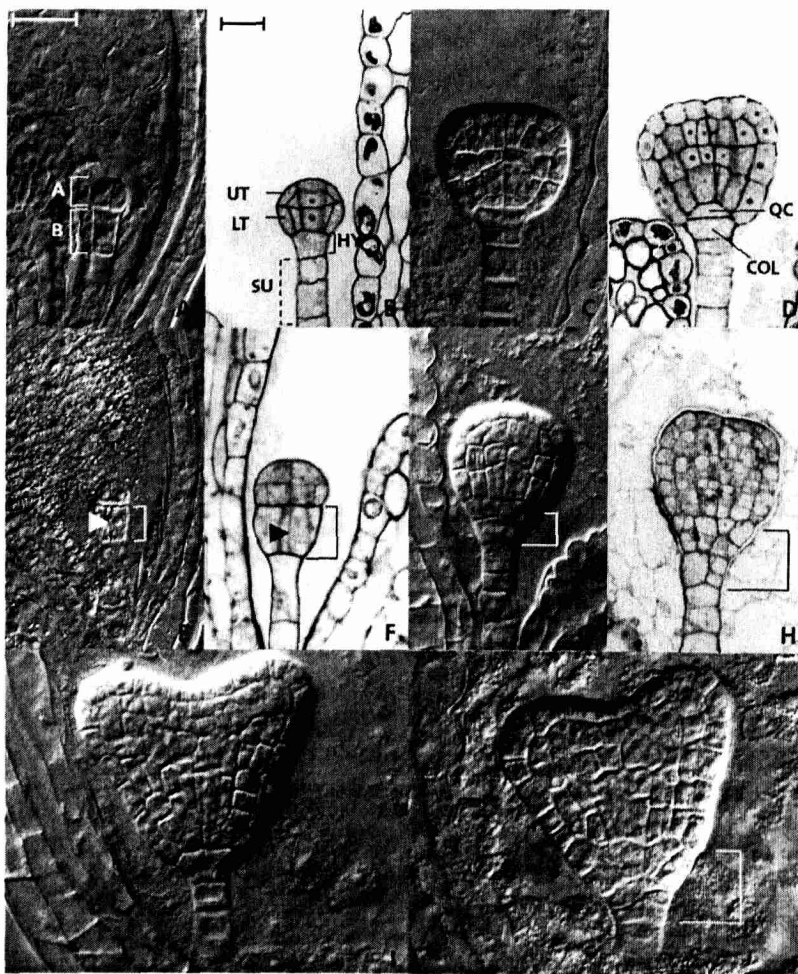
Roots can be regenerated in tissue culture, in a context entirely different from *in planta* root formation. Callus was readily induced from *hbt* mutant tissue, which confirmed that there are no apparent general defects in cell division. *hbt<sup>2311</sup>* mutant callus gave rise to small irregular outgrowths on root inducing medium, but roots could not be regenerated. Roots could be regenerated from tissue homozygous for the Ler allele *hbt<sup>GVII-24</sup>*, but these remained very short compared to roots regenerated from wild-type tissue, and furthermore they contained no recognizable lateral root cap, all similar to the defects of primary roots from *hbt<sup>GVII-24</sup>* mutants (Fig. 9A, LRC). Roots regenerated from tissue homozygous for the weak allele *hbt<sup>es56</sup>* were longer than the *hbt<sup>GVII-24</sup>* derived roots but shorter than wild-type, and they contained normal root cap layers. We conclude that tissue culture derived roots have the same requirements for *HBT* gene function as primary or secondary roots.

Auxins promote root formation in tissue culture, and we assessed whether *hbt* mutants could be rescued by auxin addition and whether they responded to the addition of auxins. Addition of  $10^{-5}$ ,  $10^{-6}$  and  $10^{-7}$  M IAA did not rescue the *hbt* phenotype and resulted in reduction of elongation of *hbt* seedlings with similar concentration dependency as wild type

(data not shown). We conclude that auxin production or perception are not generally defective in *hbt* mutants. The auxin-inducible *AtEM101* gene (Topping and Lindsey, 1997) is not up-regulated in the *hbt* mutant background (Fig. 6C,G) so we have no evidence for auxin overproduction in *hbt* seedlings.

### DISCUSSION

Recessive mutations in the *HBT* gene give rise to seedlings that contain all major organs with correct tissue patterns including the embryonic root, but without a functional root meristem and specified root cap cells. This defect can be traced back to abnormal embryonic development. In addition, *hbt* seedlings have specific aerial phenotypes that imply additional postembryonic roles of the gene, which are not discussed here.



**Fig. 7.** Early embryogenesis in wild type (A-D,I), and *hbt<sup>5721</sup>*(E), *hbt<sup>9620</sup>*(H), *hbt<sup>G221-30/2</sup>*(J) mutants. (A,E) Quadrant stage; (B,F) octant stage; (C,D,G,H) late globular stage; (I,J) early heart stage. A, apical cell; B, basal cell; UT, upper tier; LT, lower tier; HY, hypophyseal cell; SU, suspensor; QC, prospective quiescent centre; COL, prospective columella; LRC, lateral root cap. (E,F) Arrowhead points to vertical division in hypophyseal cell progenitor. Bracketed area is the hypophyseal cell region. Bar (A,C,E,G,I,J and B,D,F,H) 25  $\mu$ m.



The *hbt* root phenotype is distinct from that of previously described mutants affecting root development, which eliminate the entire root (Berleth and Jürgens, 1993), affect the radial organization of the root (Scheres et al., 1995; Di Laurenzio et al., 1996), or affect postembryonic cell division without interfering with pattern formation (Cheng et al., 1995; Berleth et al., 1996). We have demonstrated that *HBT* gene activity is essential for (i) proper development of the hypophyseal cell region from early embryogenesis onwards; (ii) specification of the lateral root cap and a mitotically active root meristem at later stages of embryogenesis. Below we will discuss these two functions separately.

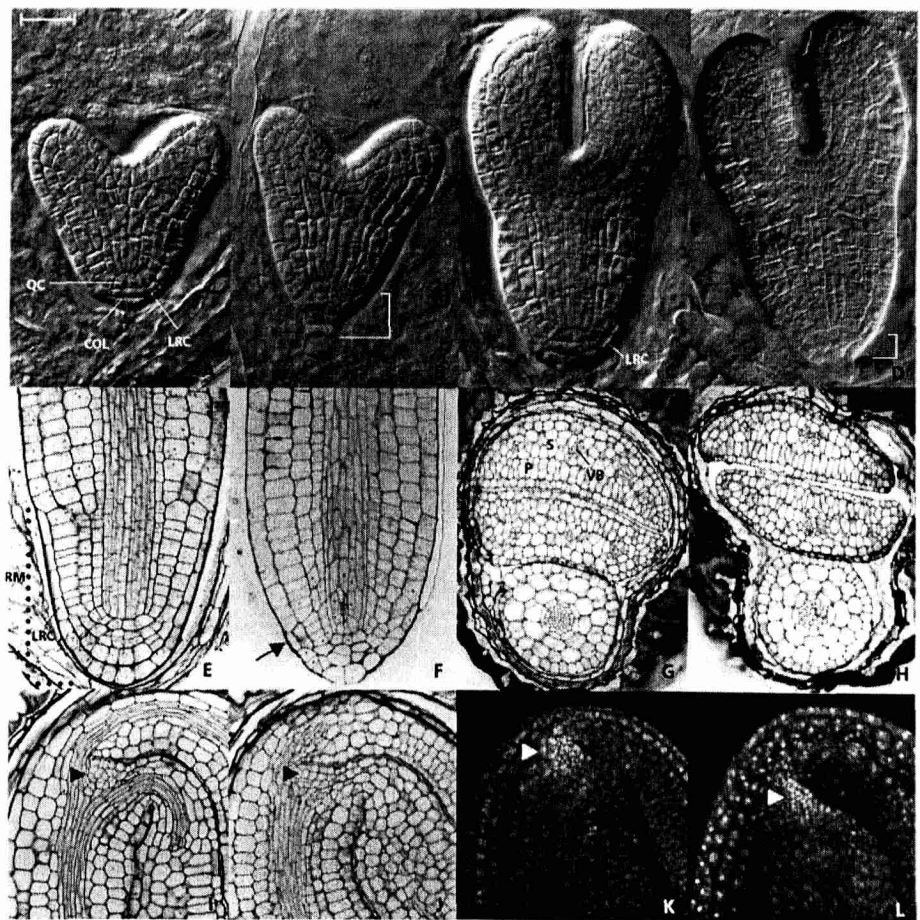
### The *HBT* gene is involved in specification of the hypophyseal cell and its progeny

The *HBT* gene product is required for proper formation of the hypophyseal cell in the embryo, and for appropriate specification of its progeny in the seedling. The most straightforward interpretation of this correlation is that the *HBT* gene is a region identity gene. Under this interpretation, the *hbt* phenotype substantiates the idea that genetic definition of the 'basal region' in the early embryo is required to direct the specification of the corresponding seedling region (Jürgens, 1995). The basal region encompasses the hypophysis which gives rise to the columella and quiescent centre (Fig. 1). The earliest deviation from normal development in *hbt* embryos occurs in the hypophyseal progenitor cell, which may suggest that this progenitor is in fact the embryonic region that first requires *HBT* gene activity. The early, strictly regional and persistent phenotype of *hbt* mutants can be considered strong support for the involvement of zygotic genes in regionalisation between the quadrant and the octant stage of embryogenesis.

An alternative explanation for the *hbt* early embryo phenotype is that the *HBT* gene product is required for the precise division pattern of the hypophysis, and that incorrect cell division interferes indirectly with later cell specification. We cannot formally exclude this possibility without early hypophyseal cell markers of known function, but we consider this explanation unlikely for two reasons. First, mutations in the *FASS* gene severely disrupt cell

morphogenesis in the embryo, including the hypophyseal cell region, but pattern formation can still occur (Torres-Ruiz and Jürgens, 1994). Specifically, columella-specific starch granules are normally present in *fs* mutants (V.W., unpubl. data). Second, the *HBT* gene is required for cell specification in secondary roots which originate from the pericycle with a completely different pattern of cell divisions. This is readily explained if the *HBT* gene serves a role in specifying the root cap and the root meristem initials rather than in hypophyseal cell division patterns.

How are early regions first defined during plant embryogenesis? The apical and basal cells result from the first zygotic division. The hypophyseal cell is formed from the basal cell at its boundary with the apical cell. It is conceivable that hypophyseal cell specification involves early interactions



**Fig. 8.** Later stages of embryogenesis in wild type (A,C,E,G,I,K) and *hbt*<sup>G221-30/2</sup> (B,D), *hbt*<sup>2311</sup> (F,H,J), *hbt*<sup>5859</sup> (L) mutants. (A,B) Nomarski optics of heart stage and (C,D) torpedo stage embryos. (E,F) Longitudinal section through embryo axis of mature embryo. (G,H) Cross sections of mature embryos displaying the radial organization of cotyledons and hypocotyl. (I,J) Longitudinal sections through the shoot apical meristem of mature embryos. (K,L) CLSM images of mature embryos stained with the nuclear stain propidium iodide. QC, prospective quiescent centre; COL, prospective columella; LRC, lateral root cap; P, palisade parenchyma; S, spongy parenchyma; VB, vascular bundle; RM, root meristem. In B,D the bracketed area is the hypophyseal cell region; in F the arrow points to skewed periclinal division in the lateral root cap; in E the dotted line indicates the root meristem region within the LRC; in I,J,K and L the arrowhead points to the shoot apical meristem. Bar 25 µm.

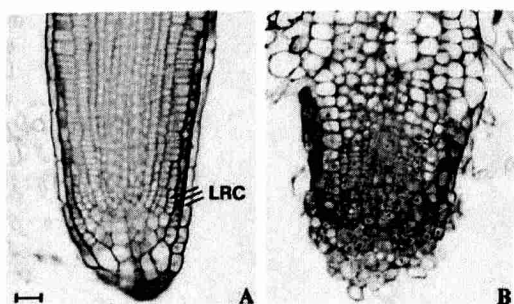


Fig. 9. Regenerated roots of wild-type (A) and *hbt*<sup>GVII-24/1</sup> (B) calli. Sections are stained with toluidine blue. LRC, lateral root cap. Bar, 25  $\mu$ m.

between the apical and basal cell (progeny). Inductive interactions to define new cell types at boundaries of early embryonic cells are well studied in animal systems. In the cellular embryo of the nematode *C. elegans*, zygotic asymmetries are translated into cellular differences concomitantly with the first cell cleavage by differential localisation or activation of transcription factors (Bowerman, 1995; Hunter and Kenyon, 1996). New cell groups are subsequently specified at the boundary of the anterior and posterior cell progeny by transmembrane signalling pathways (Evans et al., 1994; Priess et al., 1987). Thus, simple initial asymmetries give rise to a complex cellular pattern. In an analogous scenario, the *HBT* gene may either encode a cell-autonomous determinant of the basal region, or a signalling component involved in specification of this region.

Hypophyseal cell specification is also aberrant in *mp* embryos that have a defect in axialization of central region cells (Berleth et al., 1993), which may interfere with the conductivity of putative signals (Przemeck et al., 1996; Sachs, 1991). One possibility is that *MP* function in the central region is required for a signaling event that activates *HBT* gene activity in the basal region. This is consistent with double mutant analyses, which indicate that *MP* is epistatic to *HBT* in the basal region (H.W. and V.W., unpublished data). Critical tests of this hypothesis will rely on the molecular analysis of *HBT* and *MP* gene activities in wild-type and mutant backgrounds.

#### ***HBT* gene activity is required for lateral root cap specification and for meristematic activity**

At the late heart stage of embryogenesis, the defects in *hbt* mutants are no longer restricted to the hypophyseal cell region, but they encompass an immediately adjacent cell tier. In wild-type seedlings, this cell tier gives rise to the meristem initial cells with their stem cell-like division pattern, and it is the origin of the lateral root cap (Fig. 1). Cell division of meristem initials and the formation of the lateral root cap are both defective in *hbt* mutants from the heart stage onward. The extent of these defects resulting from the different alleles correlates with the extent of specification of the hypophyseal cell-derived columella root cap. If *HBT* acts cell-autonomously in the hypophyseal cell, two alternative models can account for this correlation. First, *HBT* activity may expand into the cell tier flanking the hypophysis at the heart stage of embryogenesis and influence cell fate and cell division rate in a cell-

autonomous way. In this case a pre-patterned activity in the incipient initials must regulate the expansion of the *HBT* activity domain. Second, cell fate and mitotic rate in the adjoining cells may be induced by non-autonomous, *HBT*-dependent short-range signals. There are precedences for short-range signals that influence adjoining cells in animal development, such as the hedgehog signal that is produced in *engrailed*-expressing cells (Lee et al., 1992).

Candidate mediators for the *HBT*-dependent postembryonic stimulus of root meristem activity are the *ROOT MERISTEMLESS* (*RML*) genes (Cheng et al., 1995). *rml* and other similar mutants (Berleth et al., 1996; van den Berg et al., 1997) display normal embryonic root development, but no (or very limited) postembryonic divisions occur. Therefore the corresponding genes are required to re-activate cell division within the root meristem. However, unlike in these mutants, cell division in the *hbt* root primordium is limited from the heart stage of embryogenesis onwards. Hence, although it is possible that *RML* gene activity is *HBT*-dependent, the *HBT*-dependent mitotic activity requires additional effectors during embryogenesis. It can be expected that the identification of these and other, cell-fate related, effectors will contribute to our understanding of root meristem organization during embryogenesis.

We thank Hans Bakker, Claudia van den Berg, Lenneke Broeders, Ursula Fisscher, Nicolette Quaadvlieg and Oscar Vorst for their help in harvesting EMS mutagenised M<sub>1</sub> plants; Frits Kindt, Ronald Leito, Wil Veenendaal and Piet Brouwer for photography; Dick Smit, Marjolein Kortbeek and Emy Franck for artwork; Gerd Jürgens and Ulli Mayer for thoughtful suggestions on the seedling lethal screen and for making available three *hbt* alleles; Catherine Bellini and Herman Höfte for the *e56* allele; Philip Benfey, Ursula Fisscher, Keith Lindsey and Jocelyn Malamy for marker lines; Thomas Berleth, Sacco de Vries and Philip Benfey for useful suggestions, and Claudia van den Berg, Saskia Folmer, Renze Heidstra, and Heather McKhann for critical reading of the manuscript. H. Wolkenfelt was supported by a grant from the Human Frontier Science Program Organization (RG 327/95).

#### **REFERENCES**

- Benfey, P. N., Ren, L. and Chua, N. H. (1990). Tissue-specific expression from CaMV35S enhancer subdomains in early stages of plant development. *EMBO J.* **9**, 1677-1684.
- Berleth, T. and Jürgens, G. (1993). The role of the *monopteros* gene in organising the basal body region of the *Arabidopsis* embryo. *Development* **118**, 575-587.
- Berleth, T., Hardtke, C. S., Przemeck, G. K. H. and Müller, J. (1996). Mutational analysis of root initiation in the *Arabidopsis* embryo. *Plant Soil* **187**, 1-9.
- Bowerman, B. (1995). Determinants of blastomere identity in the early *C. elegans* embryo. (1995). *BioEssays* **17**, 405-414.
- Bush, M., Mayer, U. and Jürgens, G. (1996). Molecular analysis of the *Arabidopsis* pattern formation gene *GNOM*: gene structure and intragenic complementation. *Mol. Gen. Genet.* **25**, 681-691.
- Cheng, J.-C., Seeley, K. and Sung, Z. R. (1995). *RML1* and *RML2*, *Arabidopsis* genes required for cell proliferation at the root tip. *Plant Physiol.* **107**, 365-376.
- Clark, S. E., Running, M. P. and Meyerowitz, E. M. (1995). *CLAVATA3* is a specific regulator of shoot and floral meristem development affecting the same processes as *CLAVATA1*. *Development* **121**, 2057-2067.
- Clark, S. E., Jacobsen, S. E., Levin, J. Z. and Meyerowitz, E. M. (1996). The *CLAVATA* and *SHOOT MERISTEMLESS* loci competitively regulate meristem activity in *Arabidopsis*. *Development* **122**, 1567-1575.
- Desnos, T., Orbovic, V., Bellini, C., Kronenberger, J., Caboche, M., Traas, J. and Höfte, H. (1996). *Procuste1* mutants identify two distinct genetic

- pathways controlling hypocotyl cell elongation, respectively in dark- and light-grown *Arabidopsis* seedlings. *Development* **122**, 683-693.
- Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K., and Scheres, B. (1993). Cellular organisation of the *Arabidopsis thaliana* root. *Development* **119**, 71-84.
- Di Laurenzio, L., Wysocka-Dillar, J., Malamy, J. E., Pysh, L., Helariutta, Y., Freshour, G., Hahn, M. G., Feldmann, K. A. and Benfey, P. N. (1996). The scarecrow gene regulates an asymmetric cell division that is essential for generating the radial organization of the *Arabidopsis* root. *Cell* **86**, 423-433.
- Endrizzi, K., Moussian, B., Haecker, A., Levin, J. Z. and Laux, T. (1996). The *SHOOT MERISTEMLESS* gene is required for maintenance of undifferentiated cells in *Arabidopsis* shoot and floral meristems and acts at a different regulatory level than the meristem genes *WUSCHEL* and *ZWILLE*. *Plant J.* **10**, 967-979.
- Evans, T., Crittenden, S., Kodoyianni, V. and Kimble, J. (1994). Translational control of *glp-1* mRNA establishes an asymmetry in the *C. elegans* embryo. *Cell* **77**, 183-194.
- Hunter, C. and Kenyon, C. (1996). Spatial and temporal controls target *pal-1* blastomere-specification activity to a single blastomere lineage in *C. elegans* embryos. *Cell* **87**, 217-226.
- Ingham, P. and Martinez Arias, B. (1992). Boundaries and fields in early embryos. *Cell* **68**, 221-235.
- Jürgens, G. (1995). Axis formation in plant embryogenesis: cues and clues. *Cell* **81**, 467-470.
- Jürgens, G. and Mayer, U. (1994). *Arabidopsis*. In *Embryos. Colour atlas of Development* (ed. J. Bard), pp. 7-21. London, Wolfe Publ.
- Jürgens, G., Mayer, U., Torres Ruiz, R., Berleth, T. and Miséra, S. (1991). Genetic analysis of pattern formation in the *Arabidopsis* embryo. *Development Supplement* **1**, 27-38.
- Koornneef, M. and Stam, P. (1992). Genetic Analysis. In *Methods in Arabidopsis Research*. (ed. C. Koncz, N-H. Chua and J. Schell), pp. 85-99. World Scientific Singapore.
- Kropf, D. Induction of polarity in fucoid zygotes (1997). *Plant Cell* **9**, 1011-1020.
- Laux, T. and Jürgens, G. (1997). Embryogenesis: a new start in life. *Plant Cell* **9**, 989-1000.
- Lee, J. J., von Kessler, D. P., Parks, S. and Beachy, P. A. (1992). Secretion and localized transport suggest a role in positional signaling for products of the segmentation gene *hedgehog*. *Cell* **71**, 33-50.
- Lu, P., Porat, R., Nadeau, J. and O'Neill, S. (1996). Identification of a meristem L1 layer specific gene in *Arabidopsis* that is expressed during embryonic pattern formation and defines a new class of homeobox genes. *Plant Cell* **8**, 2155-2168.
- Malamy, J. and Benfey, P. N. (1997). Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. *Development* **124**, 33-44.
- Mansfield, S. and Briarty, L. (1991). Early embryogenesis in *Arabidopsis thaliana*. II. The developing embryo. *Can. J. Bot.* **69**, 461-467.
- Mayer, U., Torres Ruiz, R., Berleth, T., Misera, S. and Jürgens, G. (1991). Mutations affecting body organization in the *Arabidopsis* embryo. *Nature* **353**, 402-407.
- Mayer, U., Büttner, G. and Jürgens, G. (1993). Apical-basal pattern formation in the *Arabidopsis* embryo: studies on the role of the *gnom* gene. *Development* **117**, 149-162.
- Natesh, S. and Rau, M. (1984) In *Embryology of Angiosperms*, (ed. B. M. Johri), Springer-Verlag, Berlin.
- Peleman, J., Boerjan, W., Engler, G., Seurinck, J., Botterman, J., Alliotte, T., Van Montagu, M. and Inze, D. (1989). Strong cellular preference in the expression of a housekeeping gene of *Arabidopsis thaliana* encoding S-adenosylmethionine synthetase. *Plant Cell* **1**, 81-93.
- Priess, J., Schnabel, H. and Schnabel, R. (1987). The *glp-1* locus and cellular interactions in early *C. elegans* embryos. *Cell* **51**, 601-611.
- Przemeck, G., Mattsson, J., Hardtke, C., Sung, Z. R., and Berleth, T. (1996). Studies on the role of the *Arabidopsis* gene *MONOPTEROS* in vascular development and plant cell axialization. *Planta* **200**, 229-237.
- Quatrano, R. and Shaw, S. (1997). Role of the cell wall in the determination of cell polarity and the plane of cell division in *Fucus* embryos. *Trends Plant Sci.* **2**, 15-21.
- Sachs, T. (1991). Cell polarity and tissue patterning in plants. *Development Supplement* **1**, 83-93.
- Scheres, B., McKhann, H., Van den Berg, C., Willemsen, V., Wolkenfelt, H., de Vrieze, G. and Weisbeek, P. (1996). Experimental and genetic analysis of root development in *Arabidopsis thaliana*. *Plant Soil* **187**, 97-105.
- Scheres, B., Wolkenfelt, H., Willemsen, V., Terlou, M., Lawson, E., Dean, C. and Weisbeek, P. (1994). Embryonic origin of the *Arabidopsis* primary root and root meristem initials. *Development* **120**, 2475-2487.
- Scheres, B., Di Laurenzio, L., Willemsen, V., Hauser, M.-T., Janmaat, K., Weisbeek, P. and Benfey, P. N. (1995). Mutations affecting the radial organisation of the *Arabidopsis* root display specific defects throughout the embryonic axis. *Development* **121**, 53-62.
- Schulz, R. and Jensen, W. (1968). *Capsella* embryogenesis: the egg, zygote and young embryo. *Am. J. Bot.* **55**, 807-819.
- Shevell, D., Leu, W.-M., Stewart Gillmor, S., Xia, G., Feldmann, K. and Chua, N.-H. (1994). *EMB30* is essential for normal cell division, cell expansion, and cell adhesion in *Arabidopsis* and encodes a protein that has similarity to Sec7. *Cell* **77**, 1051-1062.
- St Johnston, D. and Nüsslein-Volhard, C. (1992). The origin of pattern and polarity in the *Drosophila* embryo. *Cell* **68**, 201-219.
- Steeves, T. and Sussex, I. (1989). Patterns in plant development. 2nd edn. Cambridge University Press, Cambridge.
- Topping, J. F. and Lindsey, K. (1997). Promoter trap markers differentiate structural and positional components of polar development in *Arabidopsis*. *Plant Cell* **10**, 1713-1725.
- Torres-Ruiz, R. A. and Jürgens, G. (1994). Mutations in the *FASS* gene uncouple pattern formation and morphogenesis in *Arabidopsis* development. *Development* **120**, 2967-2978.
- Torres-Ruiz, R. A., Lohner, A. and Jürgens, G. (1996). The *GURKE* gene is required for normal organisation of the apical region in the *Arabidopsis* embryo. *Plant J.* **10**, 1005-1016.
- van den Berg, C., Willemsen, V., Hendriks, G., Weisbeek, P. and Scheres, B. (1997). Short-range control of cell differentiation in the *Arabidopsis* root meristem. *Nature* **20**, 287-289.
- Valvekens, D., Van Lijsebettens, M. and Van Montagu, M. (1991). *Arabidopsis* regeneration and transformation (Root Explant System). Plant tissue culture Manual **A8**.
- Vernon, D. and Meinke, D. (1994). Embryogenic transformation of the suspensor in *twin*, a polyembryonic mutant of *Arabidopsis*. *Dev. Biol.* **165**, 566-573.
- Vroemen, C., Langeveld, S., Mayer, U., Ripper, G., Jürgens, G., Van Kammen, A. and De Vries, S. (1996). Pattern formation in the *Arabidopsis* embryo revealed by position-specific lipid transfer protein gene expression. *Plant Cell* **8**, 783-791.
- Zhang, J. and Sommerville, C. R. (1997). Suspensor-derived polyembryony caused by altered expression of valyl-tRNA-synthase in the *rwn2* mutant of *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **94**, 7349-7355.