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A map of *KNAT* gene expression in the *Arabidopsis* root

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Abstract

Homeodomain proteins are key regulators of patterning during the development of animal and plant body plans. Knotted1-like TALE homeodomain proteins have been found to play important roles in the development of the *Arabidopsis* shoot apical meristem and are part of a complex regulatory network of protein interactions. We have investigated the possible role of the *knotted1*-like genes *KNAT1*, *KNAT3*, *KNAT4*, and *KNAT5* in *Arabidopsis* root development. Root growth is indeterminate, and the organ shows distinct zones of cell proliferation, elongation and differentiation along its longitudinal axis. Here we show that *KNAT1*, *KNAT3, KNAT4* and *KNAT5* show cell type specific expression patterns in the *Arabidopsis* root. Moreover, they are expressed in different spatially restricted patterns along the longitudinal root axis and in lateral root primordia. Hormones play an important role in maintenance of root growth, and we have studied their effect on *KNAT* gene expression. We show that *KNAT3* expression is repressed by moderate levels of cytokinin. In addition, we show that the subcellular localization of KNAT3 and KNAT4 is regulated, indicating post-translational control of the activities of these transcription factors. The regulated expression of *KNAT1*, *KNAT3, KNAT4* and *KNAT5* within the *Arabidopsis* root suggests a role for these genes in root development. Our data provide the first systematic survey of *KNAT* gene expression in the *Arabidopsis* root.

Abbreviations: GUS, β-glucuronidase; UAS, upstream activating sequence; ACC, 1-aminocyclopropane-1-carboxylic acid; IAA, indole-3-acetic acid; YFP, yellow fluorescent protein; GFP, green fluorescent protein; NLS, nuclear import signal; TALE, three amino acid loop extension, class of homeodomain proteins

Introduction

The tissues and organs of plants originate from meristems. These are indeterminate, self-renewing groups of cells located in the root and shoot apices. Meristems can be active throughout the lifetime of a plant and must maintain a balance between cell proliferation and differentiation. It has been shown that cell position is more important than cell lineage in determining the fate of plant cells (reviewed in Irish and Jenik, 2001; Scheres, 2001). Plant cells acquire positional information via cell–cell communication, and a network of local cellular interactions determines the

coordination and fate of cells. Genetic control underlies this process and determines the form of the organism.

The simple and regular architecture of the *Arabidopsis* root, with its indeterminate growth and transparency, have made it an ideal system for the study of cell–cell interactions (Dolan *et al*., 1993). Along the longitudinal axis of the primary root, three distinct but overlapping zones of cell development can be identified. Cells proliferate within the meristematic zone, which is located at the distal end of the root tip. In the adjacent elongation zone cells elongate rapidly before entering the differentiation zone. Cells differentiate to form a distinct and regular arrangement of different cell types. In the mature root single layers of pericycle, endodermis, cortex, and epidermis surround a central vascular bundle. Unlike in the shoot, lateral organs in the root are not formed as direct products of the apical meristem. Lateral roots are initiated from fully differentiated pericycle cells within the mature region of the root. These cells re-enter the cell cycle, divide, and establish lateral root meristems (Laskowski *et al*., 1995; Malamy and Benfey, 1997).

Ultimately, cell characteristics are defined by the set of genes transcribed in a particular cell. Transcription factors therefore play a crucial role in cell development and identity. A specific class of transcription factors, the homeodomain proteins, was first characterized in animals, where they have been shown to be key regulators of patterning during development (Gehring, 1987). Homeodomain proteins act as combinatorial switches that turn the expression of cascades of genes on and off. The DNA binding of individual homeodomain proteins is generally weak. Protein–protein interaction has been shown to be necessary to confer high affinity binding of homeodomain proteins to their cognate target sequence, and ternary homeodomain protein complexes have been found to play important roles in the combinatorial control of gene expression (Gehring, 1987). This feature makes it possible for homeodomain proteins to be involved in a series of developmental processes with different protein partners.

The first homeodomain protein to be identified in plants was KNOTTED1 (KN1) from maize (Vollbrecht *et al*., 1991). KNOTTED1 belongs to the TALE family of homeodomain containing proteins (Burglin, 1997). Members of this family can be found in fungi, animals and plants and have been shown to interact with each other to confer high DNA binding specificity (Jaw *et al*., 2000; Smith *et al*., 2002). Recently, an investigation of *Arabidopsis* TALE protein interactions revealed a heavily connected network of interactions of TALE proteins with each other and with members of the OVATE protein family (Hackbusch *et al*., 2005). The complex network suggests functional redundancy within the TALE protein family and also the possibility of compensatory interactions within the regulatory network.

The *Arabidopsis* genome contains eight *KNOTTED1*-like genes. According to their sequence similarities and their expression patterns, these genes can be divided into two subclasses. Two of the class I genes (*STM1*, *KNAT1*, *KNAT2* and *KNAT6)* have been shown to play a role in proper development of the shoot apical meristem (Lincoln *et al*., 1994; Dockx *et al*., 1995; Long *et al*., 1996; Semiarti *et al*., 2001). Plants mutant for *STM1* fail to initiate a shoot apical meristem (Long *et al*., 1996). It has also been shown that the *brevipedicellus* (*bp*) mutant of *Arabidopsis* represents a loss-of-function mutant of *KNAT1*. KNAT1/BP acts in a redundant fashion with STM1 in shoot apical meristem maintenance and also regulates inflorescence architecture (Byrne *et al*., 2002; Douglas *et al*., 2002; Venglat *et al*., 2002). In addition, misexpression of all class I genes perturbs leaf development (Lincoln *et al*., 1994; Chuck *et al*., 1996; Williams, 1998; Pautot *et al*., 2001; Dean *et al*., 2004). The roles of the class II genes *KNAT3*, *KNAT4*, *KNAT5* and *KNAT7* remain to be established.

There is increasing evidence that *KNAT* genes have roles in *Arabidopsis* root development. Northern blot data showed that *KNAT3*, *4* and *5* are expressed in roots, and a β-glucuronidase (*GUS*) gene driven by the *KNAT3* promoter exhibited expression in the mature *Arabidopsis* root (Serikawa *et al*., 1996; Serikawa *et al*., 1997). The *KNAT2* promoter has also been reported to be active in root tissue (Hamant *et al*., 2002). Moreover, Dean *et al*. showed that *KNAT6* is expressed in the phloem tissue of roots. Downregulation of *KNAT6* expression resulted in an increased number of lateral roots, suggesting that *KNAT6* is involved in lateral root formation (Dean *et al*., 2004). As a first step in understanding the role of *KNAT* genes in *Arabidopsis* root development, we have analysed the

transcriptional and post-translational regulation of *KNAT1*, *KNAT3*, *KNAT4*, and *KNAT5* in roots.

Materials and methods

Transgenic plant lines

The generation of the GAL4-GFP enhancer trap lines has been described (Haseloff, 1999). Enhancer trap lines used were Q0171, J0631, Q2393, J2613, and J2281. Lines Q0171 (N9207), J0631 (N9095), and Q2393 (N9120) are available from the Nottingham *Arabidopsis* Stock Centre (http://nasc.nott.ac.uk/). T-DNA knockout lines used were from the SALK collection (Alonso *et al*., 2003): *knat3*: Salk136464, *knat4*: Salk102021, *knat5*: Salk000339, *knat7*: Salk110899.

Construction of promoter reporter gene and overexpression plasmids

KNAT1 promoter-*GUS* construct (K1ProGUS): A 1957 bp *KNAT1* promoter fragment directly upstream of the *KNAT1* gene start codon (bp 9535–11492 of IGF-BAC F9M13, EMBL:AC006267) was PCR amplified from genomic DNA of *Arabidopsis thaliana* ecotype C24, introducing *Bam*HI sites on the 5′ and 3′ ends. The fragment was cloned into the *Bam*HI sites of pBI 101 (Jefferson *et al*., 1987).

KNAT3 promoter-*GUS* construct (K3ProGUS): A 2913 bp fragment of the region directly upstream of the *KNAT3* coding region (bp 69 581–72 494 of IGF-BAC F21J6, EMBL:AC006259) was cloned into the *Bam*HI sites of pBI101. The 2794 bp 5′ *Cla*I, *Nde*I fragment in this construct was derived by direct subcloning from plasmid pIIIC3 containing the promoter region of *KNAT3* (provided by Kyle Serikawa), the 119 bp 3′ *Nde*I, *Bam*HI fragment was derived by PCR from the same plasmid, introducing a *Bam*HI site on its 3′end.

KNAT4 promoter-*GUS* construct (K4ProGUS): A 2413 bp *KNAT4* promoter fragment including the *KNAT4* gene start codon (bp 59 138–61 550 of ESSA BAC T5K6, EMBL:AL39122) was PCR amplified from genomic DNA of *Arabidopsis thaliana* ecotype C24, introducing a *Sal*I site on the 5′ and a *Bam*HI site on the 3′ end. The fragment was cloned into the *Sal*I, *Bam*HI sites of pBI 101 (Jefferson *et al*., 1987).

KNAT5 promoter reporter gene constructs: A 1117 bp *KNAT5* promoter fragment directly upstream of the *KNAT5* gene start codon (bp 46 240–45 123 of IGF-BACF10N7, EMBL:AL021636) was PCR amplified introducing a 5′ *Bam*HI and a 3′ *Bgl*II site, filled in, and cloned into the *Hinc*II sites of pBS (Short *et al*., 1988) (resulting plasmid: pETK501). The 3′ 437 bp *Bam*HI, *Bgl*II fragment of pETK501 was then cloned into pBI101 and the 5′ 687 bp *Bam*HI fragment of pETK501 was inserted into the BamHI site resulting in K5ProGUS. The same cloning strategy was used to insert the *KNAT5* promoter fragment into *pTR 35S::mGAL4-VP16* and subsequently into ET15 (http://www.plantsci.cam.ac.uk/Haseloff), resulting in *pBIN K5::GAL4-VP16 + UAS::GFP*.

KNAT overexpression constructs: The *KNAT1*, *3, 4*, and *5* cDNAs were amplified by RT-PCR from RNA of *Arabidopsis thaliana* ecotype C24, introducing 5′ *BamH*I and 3′ *Eco*RI sites (Primers: KNAT1 5′: GGC GGA TCC AAC AAT GGA AGA ATA CCA GCA TGA CAA C; KNAT1 3′: GGC GAA TTC TGG ACC GAGACG ATA AGG TCC ATC ATC ACC C; KNAT3 5′: GGC GGA TCC AAC AAT GGC GTT TCA TCA CAA TCA TC; KNAT3 3′: GGC GAA TTC CGC GAA CCG CTC TCT TCC GCT AT; KNAT4 5′: GGC GGA TCC AAC AAT GGC GTT TCA TAA CAA TCA CTT T; KNAT4 3′: GGC GAA TTC ACG GTC TCT TCC GCT GTT TTC; KNAT5 5′: GGC GGA TCC AAC AAT GTC GTT TAA CAG CTC CCA CCT C; KNAT5 3′: GGC GAA TTC CGA CTT CCC GGT CCG TTT ACG TTT G). The cDNA fragments were cloned into a plant transformation vector derived from pBI121 (Jefferson *et al*., 1987). The vector contained a yellow fluorescent protein variant that had been constructed by introduction of the T203Y mutation (Ormo *et al*., 1996) into the *mGFP5* sequence (Siemering *et al*., 1996) by site directed mutagenesis. The *KNAT* and *YFP* coding sequences were fused in frame. The resulting plasmids were designated 35S::KNAT-YFP.

For directing the KNAT-YFP fusions to the nuclei, *YFP* was amplified by PCR from pUC18-YFP adding a nuclear import sequence to the 3′ end of the *YFP* sequence (Primers: NLSGFP3: GGC GAG CTC TTA GCG TGG GTC TTC GAC TTT TCT CTT CTT CTT TGG TTT GTA TAG TTC ATC CAT GCC, GAL4-GFP5: GCC

GAA TTC AGT AAA GGA GAA GAA CTT TTC), resulting in *YFP-NLS*. The *YFP* gene in the *35S::KNAT-YFP* constructs was then replaced by *YFP-NLS* via the *Eco*RI, *Sac*I sites, resulting in the *35S::KNAT-YFP-NLS* constructs.

For the generation of overexpression constructs that did not contain *YFP*, the *YFP* gene in the *35S::KNAT-YFP* plasmids was replaced by linkers containing either a stop codon or a nuclear import sequence, resulting in the *35S::KNAT-NLS* or *35S::KNAT-S* constructs (Linker introducing stop codon: ESTOPPS-1: AAT TCT AAC TGC AGA GCT, ESTOPPS-2: GAT TGA CGT C, linker introducing nuclear import sequence: ENLSXS-1: AAT TCC CAA AGA AGA AGA GAA AAG TCG AAG ACC CAC GCT AAT CTA GAG AGC T, ENLSXS-2: CTC TAG ATT AGC GTG GGT CTT CGA CTT TTC TCT TCT TCT TTG GG).

Growth conditions and treatments

Plants were germinated and grown under a 16 h light, 8 h dark photoperiod on media containing 0.5 × Murashige and Skoog salt mixture (MS), 0.5 g/l 2-(*N*-morpholino) ethanesulfonic acid (MES) pH 5.7 and 0.7% agar. For hormone treatments the media were supplemented with 0.1 or 1 μM kinetin (SIGMA), 5 or 50 μM 1-amino-cyclopropane-1-carboxylic acid (ACC, SIGMA) or gibberellic acid (DUCHEFA) in a concentration range of 100 μM–10 nM. For auxin treatment plants were grown for 5 days on vertical plates (10 cm × 10 cm) on media that contained 1.2% phytagel (SIGMA) instead of agar. For lateral root induction plates were flooded with 10 ml of a 1 μM indole-3-acetic acid (IAA, SIGMA) solution, left for 15 min, rinsed with water and returned to their vertical position. For kinetin induction plants were grown for 6 days on media without kinetin. They then were transferred into liquid media (0.5× MS, pH 5.7) containing 0 or 1 μM kinetin.

Arabidopsis *transformation*

Arabidopsis thaliana ecotype C24 was transformed by root transformation or floral dip. Plasmids were electroporated into *Agrobacterium tumefaciens* GV3101 (Koncz and Shell, 1986) for floral dip or LBA4404 (Ooms *et al*., 1982) for root transformation. Root transformation was performed according to Valvekens *et al*. (1988). Floral dip was performed as described (Clough and Bent, 1998). Transgenic plants were selected on media containing 50 mg/l kanamycin.

RT-PCR

For analysis of *KNAT* transcript levels in hormone treated plants, plants had been grown on liquid hormone-free media (0.5× MS, pH 5.7) for 6 days and then transferred to media containing no hormone, 50 μm ACC, or 1 μM kinetin. Roots were harvested 2, 4, 6, and 24 h after the transfer. RNA was isolated from roots and cDNA was generated from the RNA. PCR was performed with primers that bound to the coding region of *KNAT3*, *KNAT5*, or actin. All primers discriminated between cDNA and genomic DNA. In order to achieve linear amplification the optimal number of cycles was determined for each gene.

GUS expression assays

The T2 and T3 generation of promoter-*GUS* plants was analysed. Tissue was immersed in a solution containing 0.25 mg/ml 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc), 0.2% Triton X-100, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide and 50 mM sodium phosphate pH 7.5. Samples were then subjected to vacuum for 5 min and placed at 37 °C for up to 2 days. Tissue was cleared with 70% ethanol and analysed in 50% glycerol or 25% glycerol/50% chloral hydrate using Nomarski microscopy.

Tissue preparation and confocal laser scanning microscopy

For propidium iodide staining of live cell walls, roots were submerged in 5 mg/l propidium iodide for 1 min. For pseudo-Schiff staining tissue was fixed over night in 50% methanol, 10% acetic acid, washed with water, and then treated with 1% periodic acid for 30 min. The tissue was washed again, subjected to a 2 h treatment with Schiff reagent (1.9 g sodium metabisulphite in 0.54% HCl) containing 0.1 mg/ml propidium iodide. The tissue was then cleared in chloral hydrate (80 g in 30 ml of water) and mounted on slides in Hoyer's solution (30 g gum arabic, 200 g chloral hydrate, 20 g glycerol, 50 ml water). Confocal laser

scanning microscopy was performed using a Leica TCS NT/SP microscope. Excitation wavelengths were 488 nm for propidium iodide and GFP, and 514 nm for YFP.

Results

Morphological and genetic markers of Arabidopsis *root cell differentiation*

The *Arabidopsis* root is comprised of several developmental zones (Dolan *et al*., 1993). Figure 1A–J shows roots of *Arabidopsis thaliana* ecotype C24, 4 days after germination. The meristematic region lies at the distal end of the root tip, and is comprised of small essentially isodiametric cells overlaid by the root cap. The repeated anticlinal division of cells in this zone allows growth of the separate radially arranged files of the root. The end of the lateral root cap can be used as a morphological marker for the proximal end of the meristematic zone and it also marks the beginning of the elongation zone. Cells commence rapid elongation immediately adjacent to the end of the lateral root cap at about 250 μm from the root tip (Figure 1G–J). As cells continue to elongate, obvious markers for differentiation become visible. For example, root hairs become visible as trichoblasts differentiate within the epidermal layer (Figure 1F–H). The elongation zone extends to around 600–900 μm from the root tip (Figure 1G–J). Therefore cells are still elongating at the distal end of the differentiation zone (marked by the outgrowth of root hairs from epidermal cells). These observations are consistent with previous measurements (Okada and Shimura, 1990; Ishikawa and Evans, 1995; Baluska *et al*., 1996; Rutherford and Masson, 1996; Mullen *et al*., 1998).

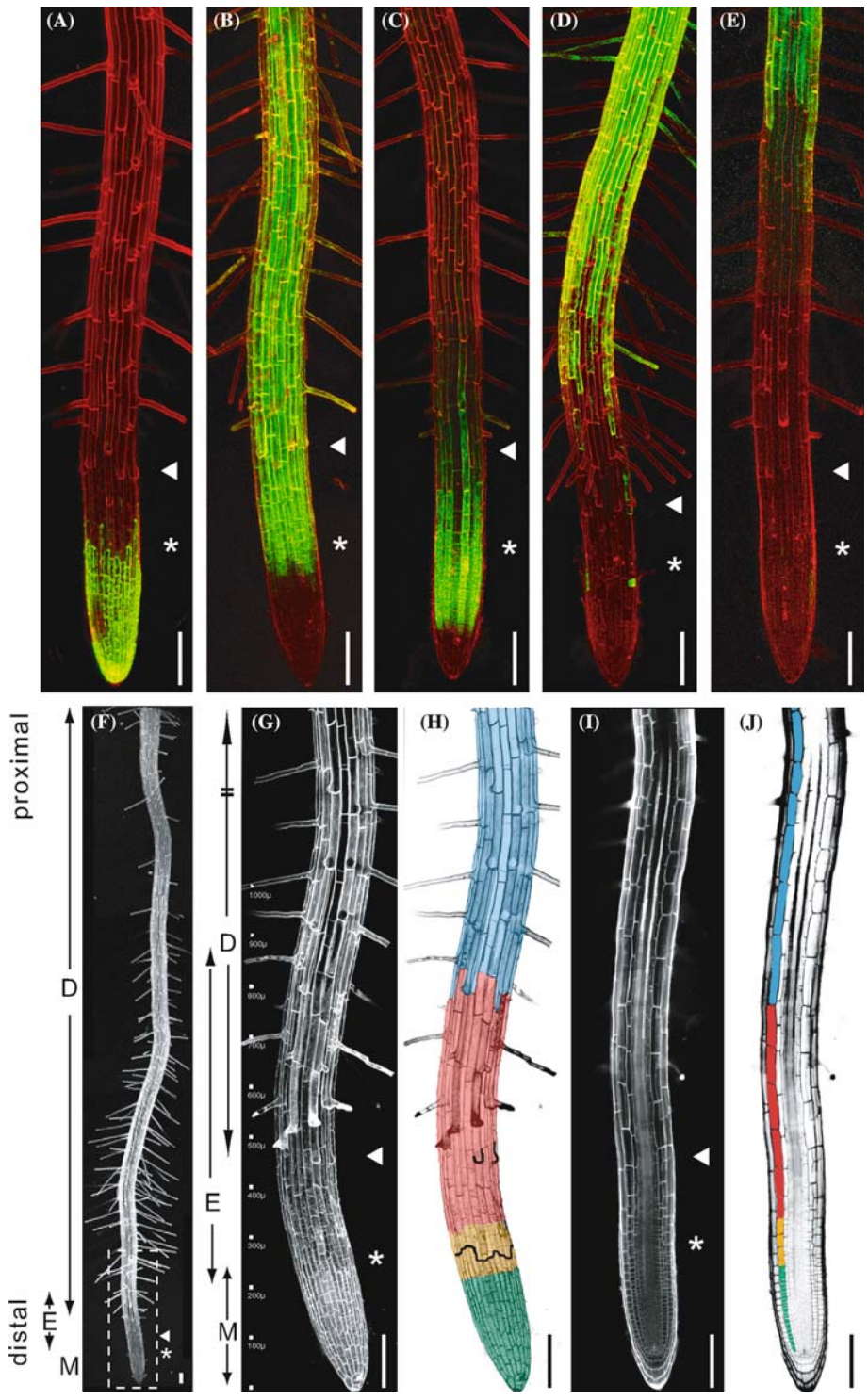
In addition to the morphological markers described above, genetic markers provide indicators for different stages of cell development in the root. In order to obtain precise markers for the developmental zones along the longitudinal axis of the *Arabidopsis* root, we screened the expression of *GFP* in *Arabidopsis* C24 enhancer-trap lines (Figure 1A–E). Different patterns of gene expression are seen to mark zones of cells at various positions within the root. In line Q0171 (Figure 1A) cells of the root cap express the *GFP* marker gene. We found that cell division in the outer layers of the meristem occurs exclusively in cells underlying the lateral root cap (S.H. and J.H., unpublished results). A change of cell fate in cells that have left the meristematic zone underlying the lateral root cap can be seen in line J0631 (Figure 1B): *GFP* expression in this line starts precisely at the end of the lateral root cap. Complementary expression patterns can also be seen in line Q2393 (Figure 1C) and line J2613 (Figure 1D). In both of these lines *GFP* expression marks the boundary between elongation and differentiation. It either ceases (Q2393) or begins (J2613) shortly after root hairs begin to emerge from the epidermis. Line J2281 (Figure 1E) shows that different degrees of differentiation can be seen in the mature root: Although no morphological change is obvious in cells that express the GFP marker in line J2281 the set of genes expressed in these cells is evidently distinct. Three conclusions can be drawn. (i) Changes in gene expression underlie the morphologically distinct zones of the root. (ii) The boundaries between these zones are sharp, not extending beyond a few cell diameters. (iii) Cells must traverse these boundaries during normal development of the root.

KNAT genes are expressed in distinct domains and cell types of the main root

Northern blot data showed that *KNAT3*, *KNAT4* and *KNAT5* transcripts were present in the *Arabidopsis* root (Serikawa *et al*., 1996). Microarray analysis of *Arabidopsis* root RNA also indicated *KNAT1* expression in root tissue (A. Navid and J.H., unpublished results). In order to investigate the exact expression patterns of these genes during root development, promoter-*GUS* fusions for *KNAT1*, *KNAT3*, *KNAT4*, and *KNAT5* were generated and introduced into *Arabidopsis* (for details of the constructs see Materials and methods section).

KNAT promoter-*GUS* plants were analysed for GUS activity in the main root 2, 3, 4, 5 and 10 days after germination. *KNAT3*, *KNAT4*, and *KNAT5* promoters showed strong activity in distinct domains and cell types of the main root (Figure 2).

Eight independent *Arabidopsis* plant lines transgenic for a *KNAT3* promoter-*GUS* fusion were analysed. The results obtained for the *KNAT3* promoter-*GUS* plants were in agreement with previously published data (Serikawa *et al*.,



1997). *KNAT3* promoter driven *GUS* expression in primary and mature secondary roots was strong in the mature part of the root and started between day 2 and day 3 after germination (Figures 2A and 5D, L). In seedling roots, GUS activity was observed in cells that had entered the differentia-

Figure 1. Zones along the longitudinal axis of 4-day-old *Arabidopsis* roots. (A)–(E) Enhancer trap line *GFP* expression patterns (shown in green): (A) line Q0171, (B) line J0631, (C) line Q2393, (D) line J2613, (E) line J2281. (F) Surface projection of a root grown on standard hormone-free 0.5×MS media. (G) Magnification of the insert shown in (F). (H) Model of developmental root zones superimposed on root shown in (G). The meristematic zone is coloured green. The distal elongation zone consists of cells that have not elongated more than 25 μm and is coloured yellow. The main elongation zone is coloured red and overlaps with the differentiation zone (begin of the outgrowth of root hairs). The end of the differentiation zone (after cell elongation has stopped) and beginning of the mature root are coloured blue. (I) Longitudinal optical section through a root tip. (J) Model of developmental root zones superimposed on root shown in (I). The colour coding is as described in (H). Roots were propidium iodide stained (shown in white in (F), (G), (I), black in (H), (J), red in (A)–(E)) and viewed with the confocal laser scanning microscope. The end of the lateral root cap is marked with an asterisk; the beginning of the outgrowth of root hairs is marked with an arrowhead. M: meristematic zone, E: zone of elongation, D: zone of differentiation and mature root. Scale bars: 100 μm.

tion zone, approximately 700–800 μm from the root tip (Figure 2A). The strongest GUS activity was observed in the pericycle, endodermis, and cortex. Epidermal cells towards the distal end of the differentiation zone also exhibited GUS activity (Figure 2G). Phloem cells occasionally showed weak GUS staining. *KNAT3* promoter driven *GUS* expression was strictly excluded from lateral root primordia (Figure 2M).

KNAT4 promoter driven *GUS* expression could be seen in the phloem, pericycle and endodermis of the root. Eleven independent plant lines transgenic for a *KNAT4* promoter-*GUS* fusion were analysed. Expression was seen at the beginning of the elongation zone where it was restricted to the phloem and the pericycle cells above the phloem poles (Figure 2C, H). Weak *GUS* expression was observed in these cell types in the mature root. In the proximal mature root *KNAT4* promoter driven *GUS* expression was strong in the endodermis (Figure 2B, I). The expression pattern in older lateral roots was similar to that of the primary root.

Eight independent *Arabidopsis* plant lines transgenic for a *KNAT5* promoter-*GUS* fusion were analysed. *KNAT5* promoter driven *GUS* activity in the main root could first be detected 2–3 days after germination. It was restricted to the root epidermis and was strongest in the distal elongation zone. *GUS* expression was activated in epidermal cells shortly before they began to elongate rapidly at the end of the lateral root cap (Figure 2E). Lower levels of activity were seen in epidermal cells of the proximal elongation zone and in those of the differentiation zone. Weak GUS activity could also be seen in the epidermis of mature roots and of the hypocotyl. Mature lateral roots showed the same *KNAT5* promoter driven reporter gene expression pattern as that of the main root. In addition, weak reporter gene activity could be seen at their bases (Figure 2D).

In summary, *KNAT* promoters were active in distinct and partly overlapping domains of the *Arabidopsis* primary root (Figure 2F, J, K). Along the longitudinal axis of the main root *KNAT* promoter activity reflected the developmental zonation of the root. *KNAT4* and *KNAT5* promoter activity was found in the elongation and differentiation zone, and in the mature root (Figure 2B–E). *KNAT3* promoter activity was only seen in fully elongated cells of the mature root (Figure 2A). *KNAT4* promoter-*GUS* expression in the endodermis started even later in the mature root, above the first initiation of lateral root primordia. In transverse root sections, *KNAT3* promoter activity was strong in pericycle, endodermal and cortical cells (Figure 2G). *KNAT4* promoter activity overlapped the domain of *KNAT3* promoter activity but was not seen in cortical cells (Figure 2H, I). *KNAT5* promoter activity was restricted to the epidermal cell layer (Figure 2D, E).

KNAT genes are expressed in adjacent domains of lateral root primordia

T2 and T3 promoter-*GUS* plants were also analysed for *KNAT* promoter driven GUS activity in lateral root primordia 10 days after germination.

Six out of ten independent *KNAT1* promoter-*GUS* transformants were found positive for GUS-staining in lateral root primordia. Shoot apical meristem staining was consistent with data obtained by Lincoln *et al.* (1994) (supp. Figure 6A). In addition, GUS-staining could be detected at the bases of lateral roots (Figure 2L, supp. Figure 6B–F). GUS activity could be seen first around stage IV of lateral root development (Malamy and Benfey, 1997) when the lateral root consisted of 4 cell layers. At this stage GUS activity was restricted to the peripheral cells that have divided only once to give rise to two cell layers (supp. Figure 6C). Cells at the

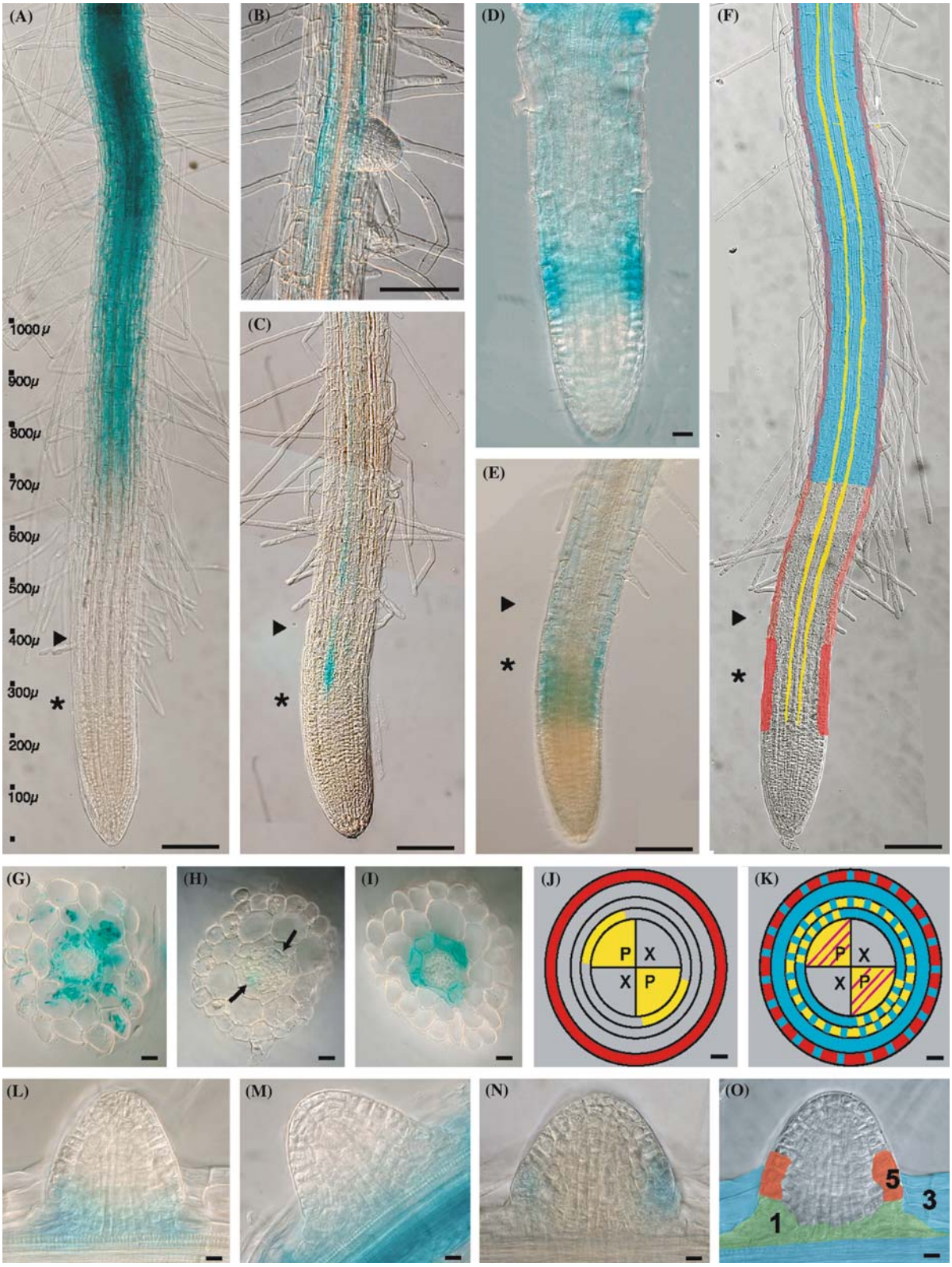

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Figure 2. Comparison of *KNAT1*, *KNAT3, KNAT4*, and *KNAT5* expression. (A)–(K) Expression of *KNAT3, KNAT4*, and *KNAT5* in 4-day-old roots. (A) *KNAT3* promoter driven *GUS* expression in the mature root. (B) *KNAT4* promoter driven *GUS* expression in the mature root. (C) *KNAT4* promoter driven *GUS* expression in the root tip. (D) *KNAT5* promoter driven *GUS* expression in a mature lateral root. (E) *KNAT5* promoter driven *GUS* expression in the root tip. (F) Model showing the expression patterns of *KNAT3, KNAT4*, and *KNAT5* in the main root. (red: *KNAT5*, blue: *KNAT3*, green: *KNAT1*) (G) *KNAT3* promoter driven *GUS* expression in a section through a mature root. (H) *KNAT4* promoter driven *GUS* expression in a section through the root elongation zone. (I) *KNAT4* promoter driven *GUS* expression in a section through a mature root. (J) Schematic of a section through the elongation zone of an *Arabidopsis* root. Cell-type specific *KNAT* gene expression is shown. For explanation of colour coding see (F); X: xylem, P: phloem. (K) Schematic of a section through a mature *Arabidopsis* root. Cell-type specific *KNAT* gene expression is shown in red for *KNAT5*, blue for *KNAT3*, yellow for *KNAT4*, and purple for *KNAT6*. (L)–(O) Expression of *KNAT1*, *KNAT3* and *KNAT5* in adjacent zones of emerged lateral roots. (L) *KNAT1* promoter driven *GUS* expression at the base of lateral roots. (M) *KNAT3* promoter driven *GUS* expression outside the lateral root primordium. (N) *KNAT5* promoter driven *GUS* expression at the flanks of the lateral root primordium. (O) Model showing the patterns of *KNAT1*, *KNAT3* and *KNAT5* promoter-GUS activity in lateral root primordia. Colour coding as in (F). The end of the lateral root cap is marked with an asterisk; the beginning of root hairoutgrowth is marked with an arrowhead. Scalebars: 10 μm in (L)–(O), (D), and (G)–(K), 100 μm in (A)–(C), (E), and (F).

base of the lateral root continued to stain throughout the development of the lateral root, resulting in a ring of *GUS*-expressing cells at the base of the lateral root primordia and of the mature lateral root (Figure 2L, supp. Figure 6D–F). Occasionally, weak staining could also be observed in pericycle cells of the main root.

KNAT3 promoter activity was seen in cells around lateral root primordia but was strictly excluded from lateral root primordia from early stages onwards (Figure 2M). Even at later stages of lateral root development when *KNAT3* promoter driven *GUS* expression was strong in the differentiation zone of the lateral root, a subset of cells at the base of the lateral roots were negative for GUS activity (Figure 5D). *KNAT4* promoter driven GUS activity was not seen in or around lateral root primordia.

KNAT5 promoter driven reporter gene activity, on the other hand, was detected in lateral root primordia (Figure 2N, supp. Figure 7). GUS activity in lateral root primordia was first seen when the lateral root primordia consisted of two cell layers (supp. Figure 7B). This corresponds to stage II of lateral root development (Malamy and Benfey, 1997). At young stages, lateral root primordia cells exhibited GUS activity throughout the primordium. At stage VI of lateral root development, when the primordium penetrated the epidermis of the main root, GUS activity was restricted to the flanks of the primordium (Figure 2N, supp. Figure 7C).

Taken together, the *KNAT* genes were expressed in adjacent but overlapping domains of lateral root primordia (Figure 2O). While *KNAT3* expression was excluded from the developing lateral root primordium from very early stages onwards, *KNAT5* was expressed in the young lateral root primordium and later in the newly developing elongation zone of the lateral root. *KNAT1* expression in the lateral root was positioned between the areas of *KNAT3* and *KNAT5* expression in the peripheral cells of the young primordium and later at the base of the lateral root.

KNAT5 expression in the epidermis marks the boundary between cell division and elongation

As homeodomain proteins can be key regulators of patterning during development, morphological events can often be correlated with precisely regulated spatiotemporal patterns of homeodomain gene expression. Promoter activity of *KNAT* genes in the *Arabidopsis* root confirmed this observation. *KNAT5* promoter-*GUS* plants showed GUS activity in the root epidermis. Activation of the *KNAT5* promoter in epidermal cells seemed to be correlated with the transition of epidermal cells from division to elongation. To investigate this in more detail, a second reporter construct (*pBIN KNAT5::GAL4-VP16 + UAS::GFP*) was generated using *GFP* as marker gene. In this construct the *KNAT5* promoter fragment was used to drive expression of the yeast transcription factor gene *GAL4-VP16*. The construct also contained the coding sequence for an endoplasmic reticulum (ER) localized variant of green fluorescent protein (mGFP5ER) under control of a GAL4 responsive upstream activation sequence (UAS). *GAL4-VP16* expressed under control of the *KNAT5* promoter drives expression of *mGFP5ER*. This provides

amplification of reporter gene expression enhancing the activity of weaker promoters.

Nine *Arabidopsis* lines transgenic for *pBIN KNAT5::GAL4-VP16 + UAS::GFP* were analysed. *GFP* expression was monitored 2, 3 and 5 days after germination. Marker gene expression was qualitatively similar to the *KNAT5* promoter-*GUS* plants. GFP fluorescence was restricted to the root epidermis and was first seen in epidermal cells that exceeded a length of 12 μm (Figure 3A). In the *Arabidopsis* root epidermis meristematic cells do not exceed a length of 12 μm. Epidermis cells stop dividing and start elongating up to a length of 25 μm underneath the lateral root cap (S.H. and J.H., unpublished results) (Figure 3B). The onset of *KNAT5* promoter driven *GFP* expression therefore marked precisely the boundary between dividing and elongating epidermal cells.

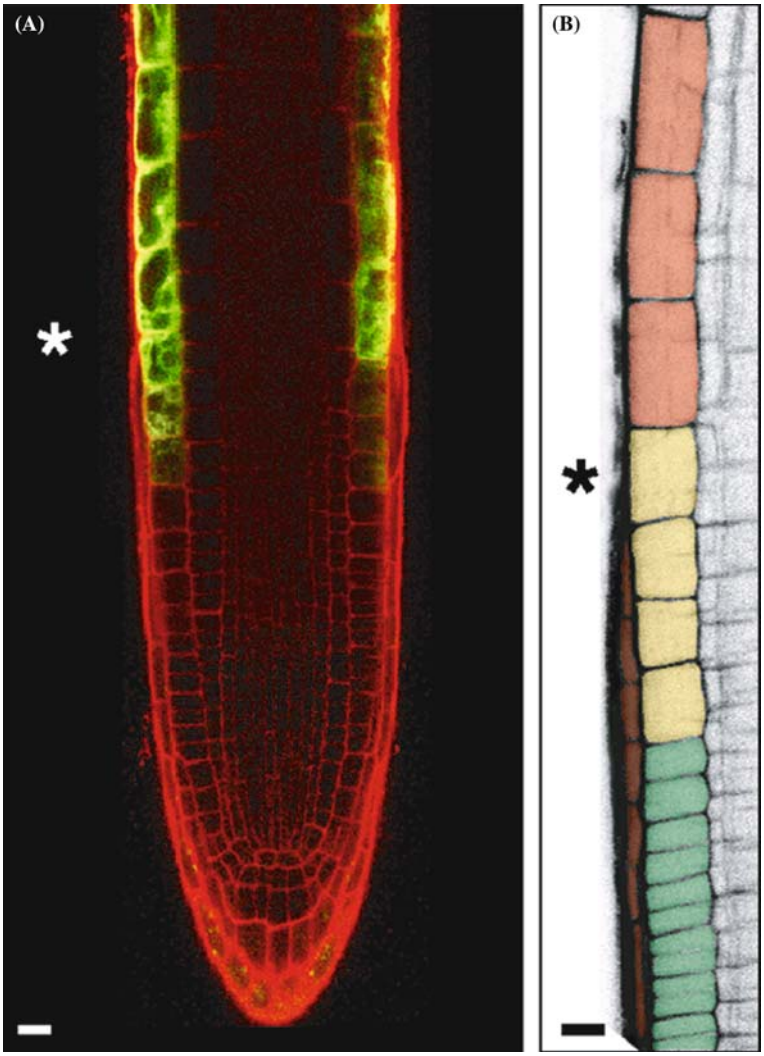

Figure 3. Expression of *GFP* by *KNAT5* promoter driven *GAL4-VP16* expression. (A) Expression of *GFP* by *KNAT5* promoter driven *GAL4-VP16* expression in a 3-day-old *Arabidopsis* root. *GFP* expression (green) is clearly and exclusively seen in the root epidermis and starts underneath the end of the lateral root cap. Cell walls are stained with propidium iodide (red). The picture was taken with a confocal laser scanning microscope. (B) Detail of root epidermis showing the transition from meristematic to elongating root cells. Root was propidium iodide stained and viewed with the confocal laser scanning microscope. Cells are coloured in according to tissue type and cell size – brown: lateral root cap cells, green: meristematic cells (cells <12 μm), yellow: elongating cells (cells between 12 and 25 μm), red: rapidly elongating cells (cells >25 μm). The end of the lateral root cap is marked with an asterisk. Scalebars: 10 μm.

KNAT protein localization is developmentally regulated

The subcellular localization of some TALE homeodomain proteins has been shown to be regulated (Hackbusch *et al*., 2005; Bhatt *et al*., 2004). In order to examine the subcellular localization of the KNAT transcription factors in the root, the cDNAs of *KNAT1*, *KNAT3, KNAT4* and *KNAT5* were translationally fused to the gene of a yellow variant of green fluorescent protein (*YFP*) and expressed in *Arabidopsis* plants under control of the constitutive *35S* promoter (Odell *et al*., 1985). Multiple independent transformed lines were obtained for the *35S::KNAT1*-YFP (n = 18), *35S::KNAT3*-YFP (n = 9), *35S::KNAT4-YFP* (n = 12) and *35S::KNAT5-YFP* (n = 14) fusions. At least three independent lines were analysed for the subcellular localization of the YFP fusion. Plants that constitutively expressed a nuclear localized *histone-YFP* gene (Boisnard-Lorig *et al*., 2001) were used for comparison (Figure 4A, F).

Localization of KNAT3-YFP and KNAT4-YFP was post-translationally regulated. In cells of the mature root KNAT3-YFP and KNAT4-YFP were found almost exclusively in the nucleus (Figure 4B, C). In the root apical meristem these protein fusions were observed in the cytosol, and were clearly excluded from nuclei (Figure 4G, H). Along the longitudinal root axis, nuclear localization began in elongating cells, and became predominant in mature cells. In contrast, the KNAT5-YFP fusion was nuclear localized in all cells of the *Arabidopsis* root (Figure 4D, J). KNAT1-YFP was also predominantly nuclear localized (not shown).

KNAT gene misexpression

The plants constitutively expressing *KNAT1-*, *KNAT3-*, *KNAT4-*, and *KNAT5-YFP* under control of the 35S promoter were analysed in the T2 and T3 generation. They did not show any obvious alterations in root and lateral root development. Since overexpression of the *KNAT* transcription factors would only be effective if they could bind to their target sequences, we sought to overcome nuclear exclusion of KNAT3- and KNAT4-YFP in the root meristem. A nuclear import signal (NLS), derived from the SV40 T-antigen (Kalderon *et al*., 1984) was fused to the 3′ end of the *YFP* coding sequence in the *35S::KNAT-YFP* constructs. Multiple transformed lines were obtained

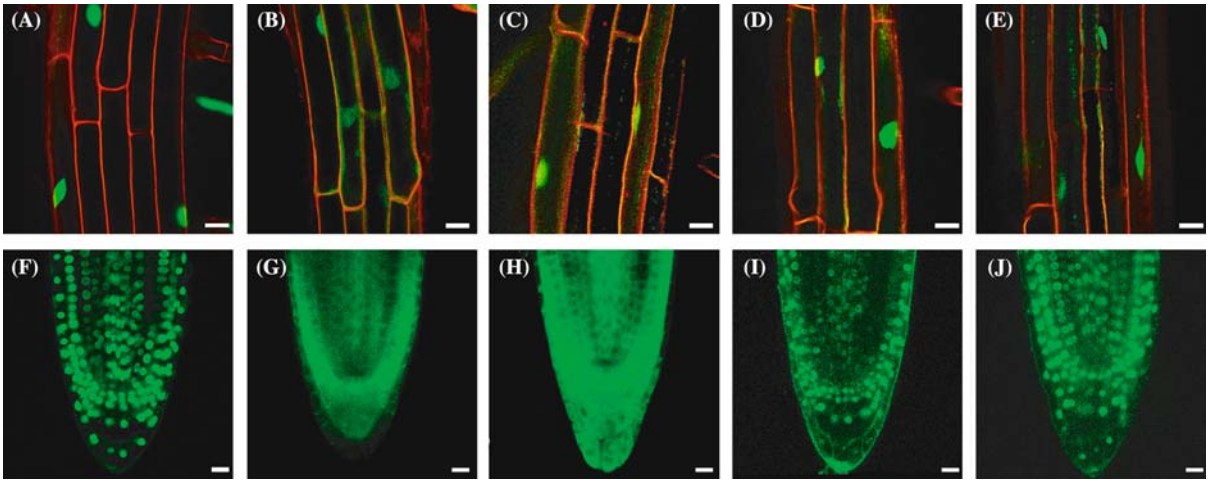

Figure 4. Subcellular localization of KNAT3, KNAT4 and KNAT5. (A)–(E) Optical section of cells of the differentiation zone of roots of 2-day-old plants. (F)–(J) Longitudinal optical section through meristems of roots of 2-day-old plants. (A), (F) Expression of *35S:Histone2B-YFP* in nuclei. (B), (G) Expression of *35S::KNAT3-YFP* showing that KNAT3 is excluded from the nuclei in the root meristem. (C), (H) Expression of *35S::KNAT4-YFP* showing that KNAT4 is excluded from the nuclei in the root meristem. (D), (I) Expression of *35S::KNAT5-YFP* in nuclei. (E), (J) Expression of *35S::KNAT3-YFP-NLS*. KNAT3 is nuclear localized in the meristem. Roots were viewed with a confocal laser scanning microscope. YFP fluorescence is shown in green. Cell walls were stained with propidium iodide (red) in (A)–(E). Scale bars: 10 μm.

for the *35S::KNAT1-YFP-NLS* (n = 20), *35S::KNAT3-YFP-NLS* (n = 6), *35S::KNAT4-YFP-NLS* (n = 8) and *35S::KNAT5-YFP-NLS* (n = 8) constructs. Adding the nuclear import sequence to the *KNAT-YFP* fusions was sufficient to localize all of the KNAT-YFP-NLS fusions to the nucleus (shown for KNAT3-YFP in Figure 4E, J). The roots of all transgenic plants had wild type morphology.

To test whether the fusion of YFP to the KNAT proteins interfered with the functionality of the homeodomain proteins, we also generated plants that constitutively expressed the *KNAT* genes without YFP and with and without an additional nuclear import signal (NLS) fused to their C-termini. Independent transformed lines were obtained for *35S::KNAT1-NLS* (n = 22), *35S::KNAT1* (n = 16), *35S::KNAT3-NLS* (n = 9), *35S::KNAT5-NLS* (n = 8) and *35S::KNAT5* (n = 12). Overexpression of *KNAT1* causes formation of lobes in leaves (Lincoln *et al*., 1994; Chuck *et al*., 1996). Severely lobed leaves were observed for all of the *KNAT1* misexpressing lines (*35S::KNAT1-NLS* (5/22), *35S::KNAT1* (5/16), *35S::KNAT1-YFP-NLS* (11/20) and *35S::KNAT1-YFP* (4/18)) (not shown). These data indicate that neither the fusion of YFP to the KNAT1 protein nor the addition of a nuclear import sequence significantly changed the frequency of the *KNAT1* overexpression phenotype. When grown on standard media, none of these lines showed significant changes in root development.

We also investigated root development in the *KNAT1* loss-of-function mutant *brevipedicellus*. In addition, T-DNA insertion lines from the SALK T-DNA knockout line collection were identified for *KNAT3*, *KNAT4*, and *KNAT5* (Alonso *et al*., 2003). Double gene knockout lines were also generated and were statistically analysed in segregating T2 populations (*knat3* × *knat4*, *knat3* × *knat5*, *knat3* × *knat7*, *knat4* × *knat5*, *knat4* × *knat7*, and *knat5* × *knat7*). No obvious alteration of main or lateral root development was found (data not shown) pointing towards functional redundancy in the TALE protein network.

Hormonal regulation of KNAT gene expression

The plant hormones ethylene and cytokinin affect cell division and differentiation. To study their effects on *Arabidopsis* root growth, plants were grown on media containing 50 μM of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) or 1 μM of the cytokinin kinetin. Root anatomy was compared to roots grown on media without any hormone supplements. Although the radial arrangement of cell types in roots grown on hormone containing media was unaltered, the developmental zones of the root along its longitudinal axis were severely affected (Figure 5). The outgrowth of root hairs from epidermal cells was observed starting from the end of the lateral root cap. This indicates that epidermal cells were not fully elongated before they started to differentiate. Consequently, the elongation zone was very short and cells in the mature root were small (Figure 5B, C). Less cells in the hormone treated roots compared to control roots also indicated that meristem size and/or cell division rate in the meristem was affected. Due to their effects on the meristem and the elongation zone, both hormone treatments therefore led to plants with morphologically similar and drastically shorter roots.

The *KNAT* promoters were active in zones of the root that were affected by hormone treatment. Therefore we decided to investigate if hormone treatment might regulate *KNAT* promoter activity. *KNAT1*, *KNAT3*, *KNAT4*, and *KNAT5* promoter-*GUS* plants were grown for 5 and 10 days on 5 μM or 50 μM ACC and subsequently assayed for GUS activity. *KNAT3* promoter-*GUS* expression was found adjacent to the end of the lateral root cap (Figure 5M). This was expected as a result of the shortening of the elongation zone in ACC treated roots. *KNAT4* promoter-*GUS* expression in the endodermis was also found closer to the root tip (not shown). Ethylene treatment therefore did not change the domain of *KNAT3* and *KNAT4* promoter activity. *KNAT5* promoter-*GUS* activity, on the other hand, increased in ACC treated roots. Epidermal GUS activity was stronger in the mature root (Figure 5G) and lateral root primordia often exhibited GUS activity throughout the primordium (Figure 5K).

Levels of *KNAT3* promoter driven *GUS* expression in roots grown on media containing 0.1 μM kinetin were significantly lower and *GUS* expression was almost undetectable in roots grown on 1 μM kinetin for 5 or 12 days (Figure 5N). In order to determine if the decrease of *KNAT3* promoter driven *GUS* expression was a direct

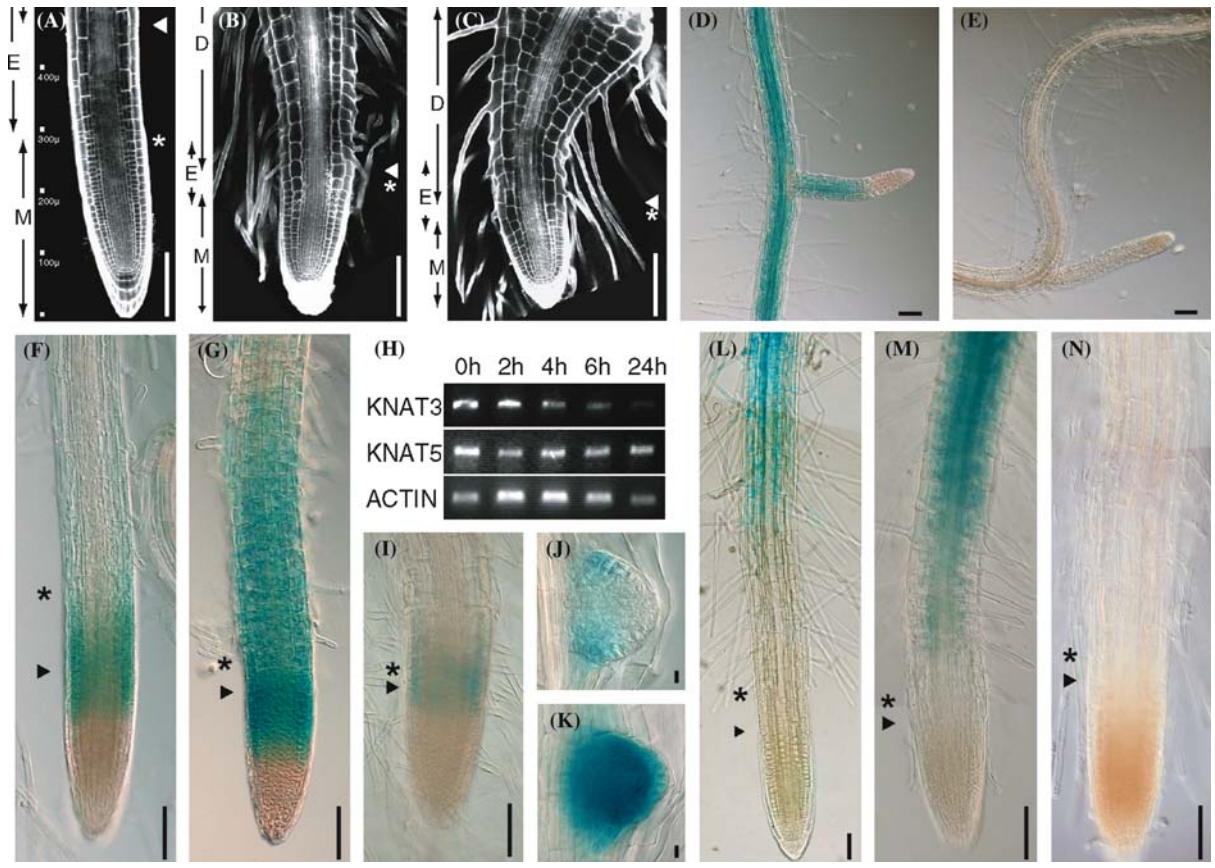

Figure 5. Hormonal regulation of *KNAT3* and *KNAT5* expression in the root. (A)–(C) Longitudinal optical sections through 4-day-old *Arabidopsis* roots grown on (A) hormone-free media, (B) 50 μM ACC, and (C) 1 μM kinetin. (D), (E), (L)–(N) *KNAT3* promoter driven *GUS* expression in (D) a 9-day-old root grown for 6 days on solid 0.5× MS media and then transferred to liquid hormone-free 0.5× MS media, (E) in a 9-day-old root grown for 6 days on solid hormone-free 0.5× MS media and then transferred to liquid 0.5× MS media containing 1 μM kinetin, (L) a 5-day-old root grown on hormone-free media (note lower magnification than in (M) and (N)), (M) a 5-day-old root grown on 50 μM ACC, (N) a 5-day-old root grown on 1 μM kinetin. Plants grown on or transferred to kinetin show no GUS activity. (H) Captures of ethidium bromide stained gels showing results of RT-PCR performed with RNA of roots that have been transferred to kinetin containing media after 6 days. PCR was performed with *KNAT3*, *KNAT5*, and actin-specific primers. RNA was isolated at 0, 2, 4, 6, and 24 h after the transfer. (F), (G), (I)–(K) *KNAT5* promoter driven *GUS* expression in (F) a 5-day-old root grown on hormone-free media, (G) a 5-day-old root grown on 50 μM ACC, (I) a 12-day-old root grown on media containing 1 μM kinetin, (J) a lateral root primordium grown on hormone-free media, (K) a lateral root primordium grown on 50 μM ACC. The end of the lateral root cap is marked with an asterisk; the beginning of root hair outgrowth is marked with an arrowhead. Scale bars: 100 μm, 10 μm in (J) and (K).

effect of the presence of kinetin in the media or an indirect effect caused by the altered morphology of the kinetin treated roots, *KNAT3* promoter-*GUS* plants were grown on hormone-free media for 6 days and then transferred to liquid media containing no or 1 μM kinetin. 36 h after the transfer, *KNAT3* promoter driven GUS activity was observed to be depressed. After 3 days of growth in media with kinetin, root morphology was not significantly altered, but *KNAT3* promoter driven GUS activity was completely abolished whereas the control plants still exhibited strong GUS activity (Figure 5D, E). We also examined *KNAT3* mRNA levels after transfer of plants to media containing kinetin. Semi quantitative RT-PCR showed that *KNAT3* transcript levels started to decrease within 4 h of exposure to kinetin (Figure 5H). This indicates that the hormone had a relatively rapid effect on *KNAT3* transcription, and that it is unlikely that the repression of *KNAT3* gene activity was due to an indirect effect of the hormone on root morphology.

The domains of the *KNAT1*, *KNAT4* and *KNAT5* promoter-*GUS* expression in primary and emerging lateral roots was not affected in plants grown on 0.1 and 1 μM of kinetin. Expression of the *KNAT5* promoter-*GUS* fusion still marked the elongation zone, which was significantly shorter due to the effects of kinetin treatment (Figure 5I).

The auxin indole-3-acetic acid (IAA) is known to induce the formation of lateral roots (Laskowski *et al*., 1995). Therefore 5 days old *KNAT1*, *KNAT3*, and *KNAT5* promoter-*GUS* plants were also subjected to treatment with 1 μM IAA. No effect on the expression of the *GUS* gene under control of the promoters of the different *KNAT* genes was observed (not shown). Gibberellic acid in a concentration range of 0.1 nM to 1 μM also did not have any effect on *KNAT* promoter activity.

Taken together, kinetin and ethylene treatment had similar effects on root morphology but they produced different effects on *KNAT* gene expression. *KNAT5* promoter activity was upregulated by ethylene treatment whereas *KNAT3* expression was repressed by kinetin. Gibberellic acid and IAA did not affect *KNAT* gene expression.

Discussion

Homeodomain gene expression in the Arabidopsis *root*

We have investigated the role of the TALE homeodomain proteins KNAT1, KNAT3, KNAT4 and KNAT5 in *Arabidopsis* root development. Homeodomain proteins are key regulators of body plan development. During organ differentiation in animal embryos, families of homeodomain genes with similar structures are often involved in a series of related developmental processes. In Drosophila embryos, for example, the identity of each segment is specified by the expression of specific homeodomain genes (Gehring, 1987; Ingham, 1988). Expression in distinct developmental zones could also be observed for families of homeodomain genes in plants. *KNOTTED1*-like TALE homeodomain protein genes are expressed in distinct zones in the tobacco shoot apical meristem (Nishimura *et al*., 1999), and *KNAT1* and *STM1* are expressed in adjacent but overlapping domains of the *Arabidopsis* shoot apical meristem (Lincoln *et al*., 1994; Long *et al*., 1996). Here we show that *KNAT1*, *KNAT3*, *KNAT4*, and *KNAT5* are also expressed in distinct developmental zones and tissues of the root (Figure 2).

Along its longitudinal axis, the *Arabidopsis* root consists of developmental domains with clear boundaries. Our analysis of *GFP* expression in *Arabidopsis* enhancer-trap lines shows that changes in gene expression underlie the morphologically distinct zones of the root (Figure 1). The activity of the promoters of *KNAT1*, *KNAT3, KNAT4* and *KNAT5* during root development was analysed by fusing them to the *GUS* reporter gene. The root meristem did not exhibit any *KNAT* promoter activity. *KNAT4* and *KNAT5* promoter-*GUS* expression started at the beginning of the elongation zone. The beginning of *KNAT5* promoter activity in the epidermis could be precisely correlated with the boundary between division and elongation in epidermal root cells. In the mature root all promoters analysed were active, marking different zones of differentiation. *KNAT6* expression was also found in the mature root. It was initiated just before the stage of lateral root formation (Dean *et al*., 2004). Microarray data suggest that *KNAT7* is also expressed in the mature root (Birnbaum *et al*., 2003).

In the lateral root primordium, expression of the *KNAT*-genes again marked different developmental areas. *KNAT3* expression was excluded from the developing lateral root primordium. *KNAT1* was expressed at the base of the primordium and *KNAT5* in the young primordium and later in the newly developing elongation zone.

Along the root's vertical axis, the *KNAT* genes showed distinct cell-type specific expression patterns. In the elongation zone the *KNAT5* promoter was active in the root epidermis whereas *KNAT4* promoter driven *GUS* expression could be seen in the phloem and pericycle cells above the phloem poles. In the mature root, *KNAT5* promoter-*GUS* expression was still seen in the epidermis where it overlapped weak *KNAT3* promoter-*GUS* expression. The *KNAT3* promoter was also active in the adjacent cortex, endodermis and pericycle. In the endodermis and pericycle it overlapped *KNAT4* promoter activity. The *KNAT4* promoter also showed weak activity in the phloem of the mature root where *KNAT6* is expressed (Dean *et al*., 2004). According to microarray data, *KNAT7* is

also strongly expressed in the stele tissue of the mature root (Birnbaum *et al*., 2003). The *KNAT2* promoter also shows activity in this tissue (V. Pautot, personal communication).

Taken together, we found that expression of the *KNAT* homeodomain genes reflects the underlying architecture of the root and the arrangement of developmental zones along the longitudinal axis of the root and in lateral root primordia. Spatially regulated patterns of gene expression correlated with distinct morphological events are characteristics of homeodomain proteins in other systems, and are consistent with a role for the *KNAT* genes in defining these regions in the *Arabidopsis* root.

Domains of KNAT gene expression

KNAT1 promoter driven *GUS* expression could be detected at the bases of lateral roots. Expression of *KNAT1* at the bases of lateral roots has been noted (Kumaran *et al*., 2002), but not analysed in detail. The development of lateral roots is a two-stage process. In *Arabidopsis*, lateral roots derive from pericycle cells above the xylem poles in the differentiation zone of the root. In the first stage of lateral root development, a primordium is formed. In the subsequent stage a meristem develops that is capable of producing a lateral root. The formation of an autonomous meristem has been shown to occur when lateral root primordia consist of at least three to five cell layers, which corresponds to stage III–V of lateral root development (Laskowski *et al*., 1995; Malamy and Benfey, 1997). *KNAT1* promoter driven *GUS* expression could be seen first around stage IV of lateral root development, and cells at the base of the lateral root continued to exhibit GUS activity throughout the lifetime of the lateral root. KNAT1 may therefore play a role after development of an autonomous lateral root meristem.

In the shoot apical meristem, *KNAT1* mRNA is localized in the peripheral zone and at the bases of leaf primordia. It has been postulated that KNAT1 is involved in mediating the switch from indeterminate to determinate growth or in maintaining cells in an undifferentiated state (Lincoln *et al*., 1994; Chuck *et al*., 1996). KNAT1 at the base of lateral roots could have a similar function. It could establish a boundary between the differentiated cells of the main root and cells differentiating according to the developmental program of the lateral root.

KNAT5 also appears to play a role in lateral root formation. *KNAT5* promoter driven *GUS* and *GFP* expression was seen in young lateral root primordia from stage II onwards and later at the flanks of the primordia. In the main root, expression of the reporter genes was restricted to the epidermis and started at the beginning of the elongation zone underneath the end of the lateral root cap. *KNAT5* promoter driven *GUS* expression in root epidermal cells preceded the actual elongation process. In the main root *KNAT5* was already expressed before cells underwent rapid elongation. Moreover, *KNAT5* promoter driven *GUS* expression was normally positioned in roots grown on cytokinin, although cells of these roots hardly elongated. It is likely that KNAT5 plays a role in the switch from meristematic to determinate cell fate in the root epidermis independent of the actual elongation process.

KNAT3 promoter driven *GUS* was expressed in primary and lateral roots in mature cells that had stopped elongating. No *KNAT3* expression could be found in the root meristem and expression was excluded from lateral root primordia from early stages onwards. In addition, KNAT3 was excluded from nuclei within meristematic cells of the *Arabidopsis* root. The expression pattern of *KNAT3*, together with the regulation of subcellular localization of KNAT3 suggest a role for the protein in the mature root. Since the formation of lateral roots requires the re-initiation of meristematic activity in differentiated root cells, a tight control mechanism that prevents KNAT3 from being active in lateral root primordia may be necessary. It is interesting in this respect that *IAA28*, a gene that suppresses lateral root development is expressed in a similar pattern to *KNAT3* (Rogg *et al*., 2001), whereas *NAC1*, a gene that promotes lateral root development, is expressed in the root tip and in lateral root primordia, areas where *KNAT3* is not expressed (Xie *et al*., 2000).

KNAT4 promoter activity started in the elongation zone and was found in the phloem and in pericycle cells above the phloem poles. Towards the base of the root, promoter activity extended into the endodermis. *KNAT2* and *KNAT6* were also found to be expressed in the root phloem (Dean *et al*., 2004, V. Pautot, personal communication) and *KNAT7* also seems to be highly

expressed in the central part of the *Arabidopsis* root (Birnbaum *et al*., 2003). For *KNAT6* it could be shown that reduced transcript levels resulted in increased lateral root formation. Therefore it was proposed that KNAT6 negatively regulates lateral root formation (Dean *et al*., 2004). We propose that KNAT4 could act in a redundant fashion together with KNAT2, KNAT6, and possibly KNAT7 to regulate lateral root development. It is worth pointing out that *KNAT4* is not expressed in the pericycle cells above the xylem poles. *KNAT4* expression, like *KNAT3* expression, is therefore absent from the cells that give rise to lateral root primordia. Like KNAT3, the KNAT4 protein is excluded from the nucleus of meristematic cells, supporting a role for KNAT4 as a negative regulator of lateral root development.

Hormonal control of KNAT gene expression

We have shown that root expression of the class II gene *KNAT3* was downregulated by cytokinin and unaffected by ethylene treatment. When plants were grown on or transferred to media containing cytokinin, *KNAT3* expression was repressed. In roots, cytokinins are formed in the tips and transported with the transpiration stream into the shoots (van Staden and Davey, 1979). It has been proposed that lateral root induction is controlled by a cytokinin gradient that declines towards the base of the primary root (Torrey, 1962). In *Arabidopsis*, expression of the gene for the *Arabidopsis* response regulator 5 (ARR5) is directly inducible by cytokinin. *ARR5* promoter-*GUS* plants showed GUS activity complementary to that seen in the *KNAT3* promoter-*GUS* plants: GUS staining was strong in root tips and at the base of lateral roots (D'Agostino *et al*., 2000). It is thus intriguing to speculate that cytokinin levels in the root directly control *KNAT3* promoter activity.

Several lines of evidence suggest a link between the class I *KNAT* genes and hormone signalling in *Arabidopsis*. Expression of *STM1*, *KNAT1*, and *KNAT2* are upregulated by cytokinin, and for *KNAT2* it has also been shown that its expression in roots and shoots was downregulated by ethylene (Rupp *et al*., 1999; Hamant *et al*., 2002). *KNAT6* promoter activity in roots showed no response to ethylene treatment but was downregulated by cytokinin.

Cytokinins and ethylene affect the balance of cell division, elongation and differentiation in the root. Both hormones inhibit root growth in a concentration dependent way (Bertell and Eliasson, 1992; Cary *et al*., 1995; Dolan, 1997; Smalle and Van Der Straeten, 1997; Le *et al*., 2001). Cytokinin lowers the number of cells found in the root meristem and in the elongation zone (Beemster and Baskin, 2000). The root elongation zone also narrows in response to ethylene (Beemster and Baskin, 2000; Le *et al*., 2001). Cytokinin actions have often been linked to ethylene production. In *Arabidopsis*, for example, the inhibition of root elongation as a response to exogenous cytokinin has been attributed to cytokinin stimulated ethylene production (Cary *et al*., 1995). We found that *KNAT3* expression in the mature root was downregulated in the presence of cytokinin, but was normal in roots grown on ethylene. We therefore can exclude the possibility that cytokinin acts through ethylene in downregulating *KNAT3* transcript levels. *KNAT5* promoter-*GUS* expression, on the other hand, was unaffected upon cytokinin treatment but its activity increased when plants were grown on ACC containing media. Therefore, although cytokinin and ethylene have similar effects on root growth, they have distinct effects on *KNAT3* and *KNAT5* promoter activity.

In our studies we distinguished between changes in expression patterns that were the direct effect of hormone treatment and changes that were caused by the altered root morphology of hormone treated roots. Although the different zones of the root were severely affected by ethylene and cytokinin treatment we found that in most cases expression of the homeodomain genes remained positioned correctly in their domains of gene expression. This is consistent with a role for the homeodomain proteins in defining these domains and regulating the expression of downstream genes.

Protein interactions

Protein–protein interaction has been shown to be crucial for homeodomain protein function. One of the characteristics of homeodomain proteins is that they bind to their target sequences with relatively low affinity. Interaction with other proteins is often necessary to confer high affinity binding of

regulatory sequences (Gehring, 1987). In Drosophila, the TALE homeodomain proteins homothorax (HTH) and extradenticle (EXD) interact with each other. Heterodimerization is not only important for their ability to bind to DNA with high affinity (Jaw *et al*., 2000), it is also necessary for the nuclear import of the transcription factors (Rieckhof *et al*., 1997): In the absence of HTH, EXD is actively exported from the nucleus (Abu-Shaar *et al*., 1999). In plants, it has been shown several times that KNAT and KNAT-like homeodomain proteins interact with each other and with TALE homeodomain proteins of the BEL1-like family to confer high DNA binding affinity (Bellaoui *et al*., 2001; Müller *et al*., 2001; Smith *et al*., 2002; Byrne *et al*., 2003; Smith and Hake, 2003; Bhatt *et al*., 2004; Chen *et al*., 2004). In addition, the recently discovered OVATE protein family regulates subcellular localization of TALE homeodomain proteins (Hackbusch *et al*., 2005).

We found that nuclear import of the KNAT3- and KNAT4-YFP fusions was regulated in root meristems. KNAT3- and KNAT4-YFP were clearly excluded from nuclei in meristematic cells while they were predominantly nuclear localized in mature root cells. The exclusion of KNAT3 and KNAT4 from the nucleus of meristematic cells would ensure that these transcription factors are not active outside their normal domain of gene expression. The effects observed were not due to the fusion of YFP to KNAT3 and KNAT4, since all KNAT-YFP fusions were of similar length and KNAT5-YFP and KNAT1-YFP could be found in the nuclei of all root cells. We can also exclude mislocalization of the fusion proteins due to their overexpression since we found all fusion proteins to be expressed at approximately the same level. It is likely that members of the OVATE protein family are responsible for the nuclear exclusion of KNAT3 and KNAT4 in meristematic cells. At present, little information is available about the tissue specific expression patterns of *OVATE* genes in *Arabidopsis*. However, it has been shown that KNAT3 and KNAT4 can interact with several members of the OVATE family in a yeast two-hybrid system (Hackbusch *et al*., 2005).

The promoters of *KNAT1*, *KNAT3, KNAT4* and *KNAT5* were all active in specific domains and cell types of the *Arabidopsis* root. Indeed, expression of the *KNAT* genes outside of these domains did not result in any obvious alterations of root development. Overexpression of *KNAT6* also was reported to have no effect on root development (Dean *et al*., 2004). Misexpression of homeodomain proteins can only be effective if the factors necessary for the binding of the homeodomain proteins to their target sequences are also expressed in the respective tissues. The addition of a nuclear import signal to the KNAT3- and KNAT4-YFP fusions, for example, was sufficient to direct them into meristematic cells of the *Arabidopsis* root. In the nucleus, however, additional factors may be necessary to confer binding to their target sequences. Likely candidates would be proteins of the BEL1-like family. In *Arabidopsis* the BEL1-like family of homeodomain transcription factors consists of at least 12 members (Becker *et al*., 2002). It has been shown that KNAT1 interacts with the BEL1-like protein BELLRINGER/PENNYWISE/VAAMANA (At5 g02030) to regulate *Arabidopsis* shoot architecture (Byrne *et al*., 2003; Smith and Hake, 2003; Bhatt *et al*., 2004). KNAT1 also regulates the nuclear entry of BELLRINGER (Bhatt *et al*., 2004). Several members of the BEL1-like family have also been shown to be able to interact with KNAT proteins in yeast two-hybrid studies (Bellaoui *et al*., 2001; Hackbusch *et al*., 2005). Little is known about the expression of *BELL* genes in *Arabidopsis*. However, *BEL1* and *BELLRINGER* transcripts have been detected in roots (Reiser *et al*., 1995; Byrne *et al*., 2003). It is likely that other members of the BEL1-like protein family are also present in the root and there can interact with KNAT proteins. Moreover, overlapping cell type specific activity of the *KNAT* promoters also makes direct interaction between the KNAT proteins possible.

TALE homeodomain proteins are part of a complex regulatory network of protein–protein interactions. For example, in a yeast two-hybrid screen KNAT5 has been shown to be able to interact with itself, with 4 different members of the OVATE family, 9 members of the BELL family, and with KNAT6 and KNAT7 (Hackbusch *et al*., 2005). Our data suggest that KNAT1, KNAT3, KNAT4, and KNAT5 play a role in root development, but that these roles are obscured by redundancy. Functional redundancy may arise not only due to overlapping function between homologous members of the gene family, but also due to compensatory interactions within the regulatory network. We found *KNAT* gene expression in the

root to be developmentally regulated and cell-type specific. Expression of the *KNAT* genes overlapped spatially and temporally. Therefore it is possible that different combinations of *KNAT* genes can be active in different cell types. It is likewise possible that the *KNAT* genes have redundant functions in specific cell types. This is especially likely for *KNAT* genes expressed in the inner cell layers of the *Arabidopsis* root. In these cell layers, KNAT2, KNAT3, KNAT4, KNAT6, and KNAT7 seem to be present. As for the TALE class of homeodomain proteins in animal systems, the *KNAT* genes appear to be involved in the combinatorial control of gene expression, whose expression is spatially regulated and post-translationally controlled. The identification of KNAT protein interaction partners expressed in the same tissue types, as well as the creation of multiple knockout plants within the *KNAT* gene family are high priorities for the further study of these genes. This work provides a map of the spatial regulation of the KNAT proteins in the root, and this will provide a basis for the selection of potential interactors.

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References

Abu-Shaar, M., Ryoo, H.D. and Mann, R.S. 1999. Control of the nuclear localization of extradenticle by competing nuclear import and export signals. Genes Dev. 13: 935–945.

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C. and Ecker, J.R. 2003. Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science 301: 653–657.

Baluska, F., Volkmann, D. and Barlow, P.W. 1996. Specialized zones of development in roots: view from the cellular level. Plant Physiol. 112: 3–4.

Becker, A., Bey, M., Burglin, T.R., Saedler, H. and Theissen, G. 2002. Ancestry and diversity of BEL1-like homeobox genes revealed by gymnosperm (*Gnetum gnemon*) homologs. Dev. Genes Evol 212: 452–457.

Beemster, G.T. and Baskin, T.I. 2000. Stunted plant 1 mediates effects of cytokinin, but not of auxin, on cell division and expansion in the root of Arabidopsis. Plant Physiol. 124: 1718–1727.

Bellaoui, M., Pidkowich, M.S., Samach, A., Kushalappa, K., Kohalmi, S.E., Modrusan, Z., Crosby, W.L. and Haughn, G.W. 2001. The Arabidopsis BELL1 and KNOX TALE homeodomain proteins interact through a domain conserved between plants and animals. Plant Cell 13: 2455–2470.

Bertell, G. and Eliasson, L. 1992. Cytokinin effects on root growth and possible interactions with ethylene and indole3acetic acid. Physiol. Plant. 84: 255–261.

Bhatt, A.M., Etchells, J.P., Canales, C., Lagodienko, A. and Dickinson, H. 2004. VAAMANA – a BEL1-like homeodomain protein, interacts with KNOX proteins BP and STM and regulates inflorescence stem growth in Arabidopsis. Gene 328: 103–111.

Birnbaum, K., Shasha, D.E., Wang, J.Y., Jung, J.W., Lambert, G.M., Galbraith, D.W. and Benfey, P.N. 2003. A gene expression map of the Arabidopsis root Science 302: 1956–1960.

Boisnard-Lorig, C., Colon-Carmona, A., Bauch, M., Hodge, S., Doerner, P., Bancharel, E., Dumas, C., Haseloff, J. and Berger, F. 2001. Dynamic analyses of the expression of the HISTONE::YFP fusion protein in arabidopsis show that syncytial endosperm is divided in mitotic domains. Plant Cell 13: 495–509.

Burglin, T.R. 1997. Analysis of TALE superclass homeobox genes (MEIS, PBC, KNOX, Iroquois, TGIF) reveals a novel domain conserved between plants and animals. Nucleic Acids Res. 25: 4173–4180.

Byrne, M.E., Groover, A.T., Fontana, J.R. and Martienssen, R.A. 2003. Phyllotactic pattern and stem cell fate are determined by the Arabidopsis homeobox gene BELLRINGER. Development 130: 3941–3950.

Byrne, M.E., Simorowski, J. and Martienssen, R.A. 2002. ASYMMETRIC LEAVES1 reveals knox gene redundancy in Arabidopsis. Development 129: 1957–1965.

Cary, A.J., Liu, W. and Howell, S.H. 1995. Cytokinin action is coupled to ethylene in its effects on the inhibition of root and hypocotyl elongation in *Arabidopsis thaliana* seedlings. Plant Physiol. 107: 1075–1082.

Chen, H., Banerjee, A.K. and Hannapel, D.J. 2004. The tandem complex of BEL and KNOX partners is required for transcriptional repression of ga20ox1. Plant J. 38: 276–284.

Chuck, G., Lincoln, C. and Hake, S. 1996. KNAT1 induces lobed leaves with ectopic meristems when overexpressed in Arabidopsis. Plant Cell 8: 1277–1289.

Clough, S.J. and Bent, A.F. 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J. 16: 735–743.

D'Agostino, I.B., Deruere, J. and Kieber, J.J. 2000. Characterization of the response of the Arabidopsis response regulator gene family to cytokinin. Plant Physiol. 124: 1706–1717.

Dean, G., Casson, S. and Lindsey, K. 2004. KNAT6 gene of Arabidopsis is expressed in roots and is required for correct lateral root formation. Plant Mol. Biol. 54: 71–84.

Dockx, J., Quaedvlieg, N., Keultjes, G., Kock, P., Weisbeek, P. and Smeekens, S. 1995. The homeobox gene ATK1 of *Arabidopsis thaliana* is expressed in the shoot apex of the seedling and in flowers and inflorescence stems of mature plants. Plant Mol. Biol. 28: 723–737.

Dolan, L. 1997. The role of ethylene in the development of plant form. J. Exp. Bot. 48: 201–210.

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.

Douglas, S.J., Chuck, G., Dengler, R.E., Pelecanda, L. and Riggs, C.D. 2002. KNAT1 and ERECTA regulate inflorescence architecture in Arabidopsis. Plant Cell 14: 547–558.

Gehring, W.J. 1987. Homeoboxes in the study of development. Science 236: 1245–1252.

Hackbusch, J., Richter, K., Muller, J., Salamini, F. and Uhrig, J.F. 2005. A central role of *Arabidopsis thaliana* ovate family proteins in networking and subcellular localization of 3-aa loop extension homeodomain proteins. Proc. Natl. Acad. Sci. USA 102: 4908–4912.

Hamant, O., Nogue, F., Belles-Boix, E., Jublot, D., Grandjean, O., Traas, J. and Pautot, V. 2002. The KNAT2 homeodomain protein interacts with ethylene and cytokinin signaling. Plant Physiol. 130: 657–665.

Haseloff, J. 1999. GFP variants for multispectral imaging of living cells. Meth. Cell Biol. 58: 139–151.

Ingham, P.W. 1988. The molecular genetics of embryonic pattern formation in Drosophila. Nature 335: 25–34.

Irish, V.F. and Jenik, P.D. 2001. Cell lineage, cell signaling and the control of plant morphogenesis. Curr. Opinion Genet. Develop. 11: 424–430.

Ishikawa, H. and Evans, M.L. 1995. Specialized zones of development in roots. Plant Physiol. 109: 725–727.

Jaw, T.J., You, L., Knoepfler, P.S., Yao, L., Pai, C., Tang, C., Chang, L., Berthelsen, J., Blasi, F., Kamps, M.P. and Sun, Y.H. 2000. Direct interaction of two homeoproteins, homothorax and extradenticle, is essential for EXD nuclear localization and function. Mech. Dev. 91: 279–291.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. 1987. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6: 3901–3907.

Kalderon, D., Roberts, B.L., Richardson, W.D. and Smith, A.E. 1984. A short amino acid sequence able to specify nuclear location. Cell 39: 499–509.

Koncz, C. and Schell, J. 1986. The promoter of the T_L-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector. Mol. Gen. Genet. 204: 383–396.

Kumaran, M.K., Bowman, J.L. and Sundaresan, V. 2002. YABBY polarity genes mediate the repression of KNOX homeobox genes in Arabidopsis. Plant Cell 14: 2761–2770.

Laskowski, M.J., Williams, M.E., Nusbaum, H.C. and Sussex, I.M. 1995. Formation of lateral root meristems is a two-stage process. Development 121: 3303–3310.

Le, J., Vandenbussche, F., Van Der Straeten, D. and Verbelen, J. 2001. In the early response of Arabidopsis roots to ethylene, cell elongation is up- and down-regulated and uncoupled from differentiation. Plant Physiol. 125: 519–522.

Lincoln, C., Long, J., Yamaguchi, J., Serikawa, K. and Hake, S. 1994. A knotted1-like homeobox gene in Arabidopsis is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. Plant Cell 6: 1859–1876.

Long, J.A., Moan, E.I., Medford, J.I. and Barton, M.K. 1996. A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of Arabidopsis. Nature 379: 66–69.

Malamy, J. and Benfey, P. 1997. Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. Development 124: 33–44.

Mullen, J.L., Ishikawa, H. and Evans, M.L. 1998. Analysis of changes in relative elemental growth rate patterns in the elongation zone of Arabidopsis roots upon gravistimulation. Planta 206: 598–603.

Müller, J., Wang, Y., Franzen, R., Santi, L., Salamini, F. and Rohde, W. 2001. *In vitro* interactions between barley TALE homeodomain proteins suggest a role for protein–protein associations in the regulation of Knox gene function. Plant J. 27: 13–23.

Nishimura, A., Tamaoki, M., Sato, Y. and Matsuoka, M. 1999. The expression of tobacco knotted1-type class 1 homeobox genes correspond to regions predicted by the cytohistological zonation model. Plant J. 18: 337–347.

Odell, J.T., Nagy, F. and Chua, N.H. 1985. Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313: 810–812.

Okada, K. and Shimura, Y. 1990. Reversible root tip rotation in Arabidopsis seedlings induced by obstacle-touching stimulus. Science 250: 274–276.

Ooms, G., Hooykaas, P.J., Van Veen, R.J., Van Beelen, P., Regensburg-Tuink, T.J. and Schilperoort, R.A. 1982. Octopine Ti-plasmid deletion mutants of *agrobacterium tumefaciens* with emphasis on the right side of the T-region. Plasmid 7: 15–29.

Ormo, M., Cubitt, A.B., Kallio, K., Gross, L.A., Tsien, R.Y. and Remington, S.J. 1996. Crystal structure of the Aequorea victoria green fluorescent protein. Science 273: 1392–1395.

Pautot, V., Dockx, J., Hamant, O., Kronenberger, J., Grandjean, O., Jublot, D. and Traas, J. 2001. KNAT2: evidence for a link between knotted-like genes and carpel development. Plant Cell 13: 1719–1734.

Reiser, L., Modrusan, Z., Margossian, L., Samach, A., Ohad, N., Haughn, G.W. and Fischer, R.L. 1995. The BELL1 gene encodes a homeodomain protein involved in pattern formation in the Arabidopsis ovule primordium. Cell 83: 735–742.

Rieckhof, G.E., Casares, F., Ryoo, H.D., Abu-Shaar, M. and Mann, R.S. 1997. Nuclear translocation of extradenticle requires homothorax, which encodes an extradenticle-related homeodomain protein. Cell 91: .

Rogg, L.E., Lasswell, J. and Bartel, B. 2001. A gain-of-function mutation in IAA28 suppresses lateral root development. Plant Cell 13: 465–480.

Rupp, H.M., Frank, M., Werner, T., Strnad, M. and Schmulling, T. 1999. Increased steady state mRNA levels of the STM and KNAT1 homeobox genes in cytokinin overproducing *Arabidopsis thaliana* indicate a role for cytokinins in the shoot apical meristem. Plant J. 18: 557–563.

Rutherford, R. and Masson, P.H. 1996. *Arabidopsis thaliana* sku mutant seedlings show exaggerated surface-dependent alteration in root growth vector. Plant Physiol. 111: 987–998.

Scheres, B. 2001. Plant cell identity. The role of position and lineage. Plant Physiol. 125: 112–114.

Semiarti, E., Ueno, Y., Tsukaya, H., Iwakawa, H., Machida, C. and Machida, Y. 2001. The ASYMMETRIC LEAVES2

gene of *Arabidopsis thaliana* regulates formation of a symmetric lamina, establishment of venation and repression of meristem-related homeobox genes in leaves. Development 128: 1771–1783.

Serikawa, K.A., Martinez-Laborda, A., Kim, H.S. and Zambryski, P.C. 1997. Localization of expression of KNAT3, a class 2 knotted1-like gene. Plant J. 11: 853–861.

Serikawa, K.A., Martinez-Laborda, A. and Zambryski, P. 1996. Three knotted1-like homeobox genes in Arabidopsis. Plant Mol. Biol. 32: 673–683.

Short, J.M., Fernandez, J.M., Sorge, J.A. and Huse, W.D. 1988. Lambda ZAP: a bacteriophage lambda expression vector with *in vivo* excision properties. Nucleic Acids Res. 16: 7583–7600.

Siemering, K.R., Golbik, R., Sever, R. and Haseloff, J. 1996. Mutations that suppress the thermosensitivity of green fluorescent protein. Curr. Biol. 6: 1653–1663.

Smalle, J. and Van Der Straeten, D. 1997. Ethylene and vegetative development. Physiol. Plant. 100: 593–605.

Smith, H.M., Boschke, I. and Hake, S. 2002. Selective interaction of plant homeodomain proteins mediates high DNA-binding affinity. Proc. Natl. Acad. Sci. USA 99: 9579–9584.

Smith, H.M. and Hake, S. 2003. The interaction of two homeobox genes, BREVIPEDICELLUS and PENNY-WISE, regulates internode patterning in the Arabidopsis inflorescence. Plant Cell 15: 1717–1727.

Torrey, J.G. 1962. Auxin and purine interactions in lateral root initiation in isolated pea root segments. Physiol. Plant. 15: 177–185.

Valvekens, D., van Montagu, M. and van Lijsebettens, M. 1988. *Agrobacterium tumefaciens* mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc. Natl. Acad. Sci. USA 85: 5536–5540.

van Staden, J. and Davey, E. 1979. The synthesis, transport and metabolism of endogenous cytokinins. Plant, Cell Environ. 2: 93–106.

Venglat, S.P., Dumonceaux, T., Rozwadowski, K., Parnell, L., Babic, V., Keller, W., Martienssen, R., Selvaraj, G. and Datla, R. 2002. The homeobox gene BREVIPEDICELLUS is a key regulator of inflorescence architecture in Arabidopsis. Proc. Natl. Acad. Sci. USA 99: 4730–4735.

Vollbrecht, E., Veit, B., Sinha, N. and Hake, S. 1991. The developmental gene Knotted-1 is a member of a maize homeobox gene family. Nature 350: 241–243.

Williams, R.W. 1998. Plant homeobox genes: many functions stem from a common motif. Bioessays 20: 280–282.

Xie, Q., Frugis, G., Colgan, D. and Chua, N.H. 2000. Arabidopsis NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. Genes Dev. 14: 3024–3036.