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RESEARCH COMMUNICATION

Shoot meristem maintenance is controlled by a GRAS-gene mediated signal from differentiating cells

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Plant shoot development depends on the perpetuation of a group of undifferentiated cells in the shoot apical meristem [SAM]. In the Petunia mutant hairy meristem (ham), shoot meristems differentiate postembryonically as continuations of the subtending stem. HAM encodes a putative transcription factor of the GRAS family, which acts non-cell-autonomously from L3-derived tissue of lateral organ primordia and stem provascularure. HAM acts in parallel with TERMINATOR (PhWUSCHEL) and is required for continued cellular response to TERMINATOR and SHOOTMERISTEMLESS (PhSTM). This reveals a novel mechanism by which signals from differentiating tissues extrinsically control stem cell fate in the shoot apex.

Results and discussion

HAIRY MERISTEM is required for meristem maintenance

The recessive hairy meristem [ham-B4281] mutation was found in a screen for meristem defects in a population of ~60,000 dTph insertions in Petunia (Koes et al. 1995). All ham mutants [100%, n = 60, stable allele hamFT-7; Fig. 3B, below] ceased organ formation during vegetative growth after initiating a variable number of leaves [9.9 ± 3.9, n = 60], whereas wild-type plants produced 17.9 ± 1.2 [n = 20] leaves before transition to flowering (Fig. 1A,C). Terminating vegetative SAMs developed a differentiated epidermis with trichomes [Fig. 1E–G], a feature normally found only on leaf primordia and subapical stem. ham axillary meristems differentiated likewise [data not shown] but without any organ formation. If axillary shoots did grow out, they arose exclusively from early vegetative nodes and terminated after several leaves. Occasionally, ham mutants developed inflorescence nodes from adventitious shoots, as judged by a nearly opposite pair of bracts, and their apices terminated with trichomes on a differentiated epidermis [Fig. 1H,I]. Flowers were rare and produced fewer, although normal, floral organs per whorl [3–5 sepals (s), 3–5 petals (p), 1–3 stamens (st), 0 carpels (c), n = 10] than wild type [invariably 5s, 5p, 5st, 2c; Fig. 1B,D]. Intact ham flowers have never been observed. Floral meristems terminated as flat structures [Fig. 1] at the cost of inner whorls.

Before termination, ham plants could not be distinguished from wild type by shoot morphology, leaf histology, or root growth [data not shown]. Thus, HAM is specific for postembryonic maintenance of all shoot and floral meristems, reflecting a shared and essential property. The differentiation of epidermis with trichomes on the SAM is a unique feature of ham, pointing toward a developmental mechanism that has hitherto not been uncovered.

The wild-type vegetative SAM contains two outer cell layers [L1 and L2, Fig. 2A] with cell divisions predomi-
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nantly in the anticlinal plane, leading to a gain in surface area. The subepidermal L2 layer shows more frequent periclinal division in the SAM’s periphery at sites of leaf initiation [Fig. 2A], which are marked by disappearance of PhSTM expression [Fig. 2B]. PhSTM was isolated in this study as a class-1 KNOX gene, most similar to tobacco NTH15 (Nishimura et al. 1999; data not shown). Its expression pattern is typical for STM-like genes (e.g., see Long et al. 1996).

In ham seedlings with 2–4 leaves, no obvious histological deviations from wild type could be observed in the SAM [data not shown], consistent with the general absence of a ham phenotype at this stage. At termination, ham was best recognized by the absence of new leaf primordia [Fig. 2C]. In L2, periclinal divisions in the central zone of the SAM were observed more frequently [Fig. 2C], and PhSTM expression was weak, although its pattern was normal [Fig. 2D]. Two weeks after overt termination, ham meristems showed differentiated cell types of epidermis (trichomes), several layers of highly vacuolated subepidermal cells, and vascular differentiation [Fig. 2F]. This histological structure is strikingly similar to the radial pattern of stem tissue underneath a wild-type SAM: epidermis, cortex, vasculature, and pith [Fig. 2E]. At this stage, PhSTM expression could no longer be detected [data not shown].

On the basis of these data and the idea that leaf initiation requires local auxin maxima whereas stem develops by default (Reinhardt et al. 2000), we conclude that ham meristems differentiate as continuations of the stem. More importantly, PhSTM expression continues for some time after meristem termination, indicating that cells at the ham apex lose the ability to respond to PhSTM but not the potential to express it in a normal pattern.

HAM encodes a GRAS protein

ham-B4281 was genetically unstable. We differentially displayed dTph1 transposon insertions using selective inverse PCR [siPCR, see Materials and Methods]. A single siPCR product fully cosegregated with the mutation [data not shown]. The ham phenotype reverted to wild type whenever excision of dTph1 restored the translational reading frame [Fig. 3B]. This shows that the siPCR product is part of HAM.

HAM cDNA contained a 721 amino acids open reading frame with strong homology with the GRAS family [Fig. 3A; Pysh et al. 1999]. The homology extended over all the conserved residues of this family, which are located primarily in the C-terminal half of the protein. GRAS proteins involved in gibberellin signal transduction share a functional amino acid motif in the N-terminal half [DELLA; Dill et al. 2001]. HAM lacks this motif. Comparative sequence analysis of a variety of GRAS proteins, ranging from gibberellin signaling proteins to root patterning factors, indicated that HAM falls into a distinct group with the putative Arabidopsis proteins AtSCL6 and AtSCL15 (SCARECROW-like, Fig. 3C). Bootstrap analysis strongly supported this group [frequency 1.00, 1000 samplings], making AtSCL6 and AtSCL15 good candidates for HAM orthologs. The dTph1 element was inserted 413 amino acids down-

Figure 1. Phenotype of ham mutants. (A) Wild-type W138 Petunia. (B) Wild-type W138 flower with the internal whorls of organs. Five stamens (yellow arrow) surround two fused carpels (green arrow). (C) ham-B4281 plant, terminated during vegetative rosette growth. (D) ham mutant flower lacking two stamens and both carpels. (E) Wild-type vegetative apex. p3, p2, and p1 = leaf primordia in order of decreasing age; m = meristem. (F) Vegetative ham apex shortly after termination. Primordium initiation has ceased. The meristem displays ectopic trichomes (arrows). (G) As in F, 2 wk after termination, the central dome has increased in size and is covered with trichomes. (H) Wild-type inflorescence apex: fm = floral meristem, im = inflorescence meristem, br = bract, se = sepal. (I) ham inflorescence apex with ectopic trichomes. br = last initiated bracts. (J) ham floral meristem, showing termination after initiation of three stamens (st). In place of carpels, a flat apex is visible with a small outgrowth [arrow]. Bars, 100 µm.

Figure 2. Histology of ham apices. (A) Wild-type vegetative meristem. The arrow indicates a periclinal division in the L2 layer of an initiating leaf primordium. (B) In situ localization of PhSTM transcript in a wild-type vegetative apex. The signal (blue) is excluded from the leaf primordia. (C) ham vegetative apex showing a cessation of organ initiation and periclinal division in the central zone [arrow]. (D) In situ localization of PhSTM transcript in a ham apex shortly after termination. (E) Transverse section of developing stem, just below a wild-type meristem [section schematized, inset]. e = epidermis, c = cortex, v = vasculature, p = pith. (F) Older ham apex in longitudinal section showing a layered structure of differentiated tissue. e, c, v, p as in E. Arrow = trichome. Bars, 50 µm.
stream from the start codon in the coding region of HAM (Fig. 3A), probably resulting in a null phenotype. Conclusive evidence for the identity of HAM was obtained by cosuppression, using the full cDNA expressed in the sense orientation from the 35S promoter in transgenic plants. One cosuppression line was selected on the basis of absence of endogenous HAM expression (Fig. 3H) and analyzed in detail. Although vegetative development was mostly normal, axillary shoots typically terminated in hairy meristems (data not shown). Interestingly, 52% of inflorescence nodes (n = 138, 14 plants) skipped one or more organs, resuming organ initiation in the node that followed (Fig. 3F,G). This is probably a weak ham phenotype, with SAM cells differentiating into stem before acquiring organ identity. The recovery of these SAMs indicates that some self-maintaining properties of the plant’s stem cell population can compensate for a partial loss of HAM function.

HAM acts non-cell-autonomously from lateral organ primordia and stem provasculature

RT-PCR detected HAM cDNA in all shoot tips and in roots but not in expanding leaves (data not shown). HAM expression was analyzed in detail by RNA in situ hybridization. In vegetative apices, HAM was expressed in deeper layers of the meristem at the presumptive site of organ initiation (Fig. 4A), as well as in the developing stem provasculature (Fig. 4B). In transverse sections, a signal was obtained in the developing primordia at least until P6 (Fig. 4C). HAM was expressed strongly in the L3-derived ground tissues in the inner part of the primordia and weakly in the main vascular bundle of older primordia (Fig. 4C). Beneath the vegetative meristem, HAM was observed in a ring that corresponds to the provascular tissue of the stem (Fig. 4D). In the inflorescence and floral meristems, similar HAM expression was detected consistently in all organ primordia and all floral whorls (Fig. 4E). In provascular tissue (Fig. 4E), Figure 4F summarizes this pattern for a vegetative meristem.

These data indicate that HAM expression from the L3 layer of the SAM may be sufficient for function in the overlying layers L1 and L2. To test this, we analyzed the HAM::ham segregation ratios for four independent revertant branches on unstable ham-B4281 homozygotes. These branches developed wild-type flowers indeterminately. Within-flower self-fertilizations from three branches gave rise to nearly normal Mendelian 3:1 (HAM:ham) patterns. However, one revertant branch did not segregate a single wild-type progeny among 80 siblings derived from the eighth and tenth flower on the branch. As gametes are derived mostly from L2 (e.g., see
This led to increasingly bushy plants that resembled wus mutants [Laux et al. 1996]. A single siPCR product cosegregated with ter [data not shown]. The predicted TER protein was highly similar to WUS [Fig. 3D], mainly in the homeobox and with conspicuous blocks of homology in the C terminus. ter could be reverted to wild type whenever excision of dTph1 restored the open reading frame [Fig. 3E]. TER gene expression patterns were identical to WUS [Fig. 5C]. dTph1 was inserted 12 amino acids downstream from the homeobox, probably resulting in a null mutation. We refer to TER as PhWUS and to its mutation as ter.

During early vegetative growth, ter ham double mutants showed an initial stop-and-go growth characteristic for ter but subsequently started to display ham phenotypes [Fig. 5F]. We compared the structure of 15 meristems of both ter single mutants and ter ham double mutants in mature plants. In ter single mutants, we found ectopic leaves and meristems on a flat apex in 13 of 15 cases [Fig. 5F]. In contrast, in ter ham double mutants, the typical ham phenotype of a trichome-covered

**Figure 4.** Expression pattern of HAM. [A] In situ localization of HAM transcript in a near median (top right inset) longitudinal section through a vegetative apex. Signal is in the developing primordia [blue arrow] and at the presumptive position of a newly initiating primordium [red arrow]. [B] As in A, with a section located more peripherally (top right inset). The signal is seen in a developing primordium [red arrow], as well as in a ring-shaped pattern that corresponds to the developing stem vasculature [blue arrow]. [C] As in A, but in a transverse section. The position of the section is indicated in the top right inset. The signal is observed in the inner ground tissues of the primordia [red arrow] and is weaker in the main vascular bundle of older primordia [grey arrow]. The blue arrow indicates HAM expression in a ring-shaped pattern that merges with primordia P1 and P0 and corresponds to provascular tissue of the differentiating stem. P6, P5, and so forth indicate the consecutive order of primordium initiation with decreasing age. [D] As in C, but at a position just below the meristem [indicated in top right inset]. HAM expression is seen as a ring that corresponds to the provascular structure of the stem. [E] HAM localization during development of the floral meristem, as exemplified for initiating petal primordia. Expression is observed in inner cell layers at the site of petal initiation [red arrow] and in subtending provascular tissue of the developing pedicel [blue arrow]. [F] Schematic representation of the HAM expression pattern as exemplified for a wild-type vegetative meristem. Bar, 50 µm.

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**Figure 5.** Relations between ham and ter. (A) Wild-type Petunia during vegetative rosette growth. [B] Wild-type shoot apical meristem (SAM) with the first two true leaf primordia. Cotyledons have been removed. (C) In situ localization of TER [PhWUS] transcripts in a wild-type vegetative apex. [D] ter-B1382 seedling. Growth has ceased after production of the first two true leaves. [E] ter seedling apex after initiation of the two first leaves. A flat, disorganized structure replaces the SAM. [F] ter apex with an ectopic meristem [arrow, stop-and-go growth]. [G] ter ham double mutant seedling. An additional leaf, compared with D, occurred with a low frequency in ter single mutants as well. [H] ter ham double mutant seedling apex. Initiation of ectopic leaves is observed [stop-and-go]. [I] ter ham double mutant apex on an older plant. The SAM displays a trichome covered surface characteristic for ham single mutants. [J] In situ localization of PhWUS transcripts in a ham mutant apex shortly after termination. The signal is essentially normal. [K] In situ localization of PhWUS transcripts in a later ham mutant apex. Expression occurs in a disorganized pattern. [L] As in K. PhWUS expression in the main apex has disappeared. In the axillary position, expression is disorganized and deeply internal. Bars: B, 25 µm; C,E,J, 50 µm; F,I, 200 µm; H,K,L, 100 µm.

*HAM acts in parallel with TERMINATOR*

*ham* has some important similarities with *wuschel* mutants of *Arabidopsis* [the meristem is not maintained in both]. To investigate the relationships between HAM and WUS, we isolated the Petunia WUS ortholog from a mutant terminator [ter-B1382]. Like wus, ter ceased shoot development after the two first true leaves [Fig. 5A,D], continuously reinitiating ectopic leaves and defective meristems from flat apices [stop-and-go growth; Fig. 5E,F]. This led to increasingly bushy plants that rarely flowered. Occasional flowers had fewer floral organs per concentric whorl [data not shown], strongly resembling wus mutants [Laux et al. 1996].
by acting from L3 of organ primordia and provascular cell maintenance also in the shoot, stem cell daughters differentiate according to positional information from surrounding cells (Stewart et al. 2000). Conversely, they may develop into lateral organs (e.g., see Byrne et al. 1995). In the root, by positional information from surrounding cells (Stewart et al. 2000).

Role of HAM in shoot meristem and stem cell maintenance

By acting from L3 of organ primordia and provascular cell maintenance also in the shoot, stem cell daughters differentiate according to positional information from surrounding cells (Stewart et al. 2000). Conversely, they may develop into lateral organs (e.g., see Byrne et al. 1995).

In root and shoot systems of plants, the differentiation and division patterns of stem cell daughters are directed by positional information from surrounding cells (Stewart and Burke 1970; van den Berg et al. 1995). In the root, initial cells for radial cell files differentiate according to positional information from surrounding cells (Stewart et al. 2000). Conversely, they may develop into lateral organs (e.g., see Byrne et al. 1995).

The full HAM coding region was amplified with Fnu DNA polymerase and primers hamtATG (5'-ATCTAGAGTTTAAGATGATTGCAATAC-3') and hamtTGA (5'-AAGATCTTCCTCCACGCCACATTCCAGC-3') from revertant branches on homozygous insertion mutants or from their progeny. DNAs were PCR amplified with ter5'-F (5'-GACGAGGCCCATATCTTCTTCACT-3') and ter3'R (5'-CCATGTGCTAAAGGATAGTACGTAC-3') from the above cDNA library.

For selective iPCR (siPCR), genomic DNA was digested with MboI, self-ligated with T4 ligase, and relinearized with SspI. Such templates were selectively preamplified using diph1-specific primers out4' (5'-GAACGGTTGCTCCTTGACC-3') and out5' (5'-GGTCAGCGCCCAAGTTGAC-3'). Out6' flanks an MboI site in diph1 and carries any of 16 possible combinations of 3'-terminal bases (NN) extending into plant DNA. Preamplifications were reamplified with out6 + NN primers and a nested diph1-out1 primer (5'-GGAAATTCGCTCGCCCGCTGCCG-3'). Products were separated on 5% native polyacrylamide gels and stained with SYBR Gold (Molecular Probes, Inc.). Candidates were cut out of the gel, eluted in water, reamplified, and sequenced. A detailed protocol is available from the authors.

To analyze footprint alleles for hamt and ter, genomic DNA was isolated from revertant branches on homozygous insertion mutants or from their progeny. DNAs were PCR amplified with hamt3' (5'-CAAGGAGGCTCTGACATGC-3') and hamt4' (5'-CTTCGAAAGAGAAGTATAAGCATTCCC-3') or terF (5'-GAAAGAGCCTTTACCTCTGCTGCTAC-3') and terR (5'-CCCTAACAGCCTTCTCCATGACTAAG-3'). Products were separated on 5% polyacrylamide gels, and bands with small size increases relative to wild type were eluted, reamplified, and sequenced.

Expression analyses

RT-PCR was performed on first strand cDNA using gene-specific primers for the 3'-untranslated region of HAM cDNA (HAM3'F, 5'-AGGTTTATCTAAATGAAAGCCAGAAGG-3'; HAM3'R, 5'-ACAGGGAAATGAGACAAGATACATCC-3') and hamt4' (5'-CTTCCAGAAAGAGTATAAGCATTCCC-3') or terF (5'-GAAAGAGCCTTTACCTCTGCTGCTAC-3') and terR (5'-CCCTAACAGCCTTCTCCATGACTAAG-3'). Products were separated on 5% polyacrylamide gels, and bands with small size increases relative to wild type were eluted, reamplified, and sequenced.

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mation was into P. hybrida W115 using Agrobacterium tumefaciens LBA4404.

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