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

Jeroen Stuurman, Fabienne Jäggi, and Cris Kuhlemeier

Institute of Plant Sciences, University of Bern, Altenbergrain 21, CH-3013 Bern, Switzerland

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 provasculature. 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.

Plants differ from animals in that they continuously form new organs (stems, leaves, or flowers) during postembryonic shoot development. This depends on the perpetuation of a shoot apical meristem (SAM) at the very summit of the growth axis. Within the SAM, a few stem cells are specified whose daughters give rise to the entire vegetative SAM at the very summit of the growth axis. This depends on the perpetuation of a shoot apical meristem (SAM) at the very summit of the growth axis. Within the SAM, a few stem cells are specified whose daughters give rise to the entire vegetative SAM at the very summit of the growth axis.

A primary question in plant development is how cells are specified whose daughters give rise to the entire vegetative SAM at the very summit of the growth axis. This depends on the perpetuation of their ad-abaxial polarities [Waites et al. 1998; Lynn et al. 1999]. Clearly, the SAM is intricately linked to its differentiating environment by non-cell-autonomous control systems, the extent and molecular mechanisms of which are important issues.

Here, we describe the GRAS gene HAIRY MERISTEM (HAM) of Petunia. HAM mediates a signal from lateral organ primordia and stem provasculature that is essential and specific for maintaining the SAM. This defines a novel pathway that links stem cell perpetuation to differentiation.

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 dThp1 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.

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ham meristems differentiate as stem

The wild-type vegetative SAM contains two outer cell layers (L1 and L2, Fig. 2A) with cell divisions predomi-
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 encodes a GRAS protein**

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.

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 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). As in B but for ter-B1382. (F) Wild-type Petunia inflorescence producing two bracts (br) and a flower per node. (G) HAM cosuppressor showing a node without bracts and flower (arrow). [H] In situ hybridization of HAM RNA on wild-type [upper] and cosuppressed [lower] floral meristems. Wild type shows a signal in the initiating petal primordia. The cosuppressor lacks this signal. Bar, 50 µm.

Figure 3. Cloning and structure of HAM and TER. [A] Protein sequence alignment of the C-terminal portion of HAM with SHR and GAI. Absolutely conserved positions are red, and conserved residues are grey (>90%), yellow (>80%), or green (>70%), based on alignments of 12 GRAS sequences as in the cladogram of C. Atypical residues at conserved positions are not colored. VHIID, PFYRE, and SAW are domains as defined in Pysh et al. (1999). Triangle = ΔTph1 insertion in ham-B4281. WT = wild-type sequence flanking the insertion in ham-B4281, wt = footprints restoring HAM function, mut = mutant footprints allele ham-F7. [B] Tree produced by neighbor joining (ClustalG software) showing the similarities of 12 GRAS sequences. Numbers indicate bootstrap frequencies of each branchpoint in the cladogram. GenBank accession nos. AtGAI (CAA75492), AtSHR (NP195480), AtPAT1 (AA73233), AtRGA (CAA75483), LeLS (AAD05242), AtSCL6 (NP191926), AtSCR (AAR06318), AtSCL15 (NP191622), ZmDB [AAL10319], TeGRM (CAB51555), OsGAI (BAA90749), and PhHAM (AF481952). [D] Full protein alignment of TER [GenBank accession no. AF481951] and WUS [GenBank accession no.CAA09986]. Conservation is given on the basis of these two orthologs only. Red residues indicate positions in the homeobox, and grey residues denote blocks of conspicuous colinearity. [E] As in B but for ter-B1382. [F] Wild-type Petunia inflorescence producing two bracts (br) and a flower per node. [G] HAM cosuppressor showing a node without bracts and flower (arrow). [H] In situ hybridization of HAM RNA on wild-type [upper] and cosuppressed [lower] floral meristems. Wild type shows a signal in the initiating petal primordia. The cosuppressor lacks this signal. Bar, 50 µm.
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]. 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. 5E,F]. 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 provascularature 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.

Stewart and Burk 1970), the wild-type phenotype of this branch was conferred by cells that were not related clonally to L2. Given the expression pattern of *HAM*, this branch was most likely a periclinal chimera with a revertant L3. Taken together, this essentially shows a non-cell-autonomous mode of action of *HAM*. Because revertant branches did not influence the mutant phenotype in other parts of the same plant, the signal must be short range.

**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,J, 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
surface was found in 10 of 15 cases [Fig. 5I]. In the other five cases, the surface was flat and lacked trichomes but showed no sign of resumption (data not shown). Thus, in early seedling stage ter ham double mutants closely resemble ter, but at later stages they behave like ham. In situ hybridization on ham single mutants showed that, at termination, PhWUS expression was essentially normal [Fig. 5J]. In later stages, we observed no expression [Fig. 5L] or a patchy pattern [Fig. 5K]. PhWUS expression in axillary ham meristems did the same [Fig. 5L].

We conclude that specification and maintenance of stem cells by HAM and WUS are largely parallel processes, although maintenance of spatially correct PhWUS expression indirectly depends on HAM. Like PhSTM, PhWUS expression continues for some time after meristem termination. Thus, cells in a differentiating ham shoot apex lose the ability to respond to PhWUS activity but not the potential to express it.

Role of HAM in shoot meristem and stem cell maintenance

By acting from L3 of organ primordia and provascular cell maintenance in the root are unknown, depends on extrinsic antidifferentiation factor(s). Whereas also in the shoot, stem cell daughters differentiate according to the above cDNA library. For selective iPCR (siPCR), genomic DNA was digested with MboI, self-ligated with T4 ligase, and relinearized with SpI. Such templates were selectively preamplified using dPhpl1-specific primers out4 (5'-GAACGGTTGTCTCCTTGAACC-3') and out6 (5'-GGTGCAGGCCCA GATTCGATCnn-3'). Out6 flanks a MboI site in dPhpl1 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 dPhpl1-out1 primer (5'-GGAATTTCCGCTCCGCGCCGCTT-3'). Products were separated on 5% native polyacrylamide gels, and bands with small size were cut out, eluted, reamplified, and sequenced. A detailed protocol is available from the authors.

To analyze footprint alleles for ham and ter, genomic DNA was isolated from revertant branches on homozygous insertion mutants or from wild type were eluted, reamplified, and sequenced. A detailed protocol is available from the authors.

Expression analyses

RT-PCR was performed on first strand cDNA using gene-specific primers for the 3'-untranslated region of ham cDNA (HAM3' (5'-AGCTTTAT CTAATGGAAGCCGAAGAGG-3') and HAM3' (5'-ACAGGGGAAATGAG CTACCC-3') or terF (5'-GAAGAGCTTTATGGATGACGTCGACGTCACC-3') and terR (5'-CGATGTCAGGAAAATCGAGTACGGACC-3'). Products were separated on 5% polyacrylamide gels, and bands with small size were eluted, reamplified, and sequenced.

Transgenics

The full HAM coding region was amplified with Phu DNA polymerase and primers hamATG (5'-ATCTAGAGTTTAAAGATGATGGAATTTAT C-3') and hamTGA (5'-AAAGATCTTTCTCACCACACCTCCTTGTCAGT-3'), digested with BglII and XbaI, and inserted in the sense orientation into a pBl121 (Clontech)-derived vector to replace the GUS cassette. Transfor-
mation was into \textit{P. hybrida} W115 using \textit{Agrobacterium tumefaciens} LBA4404.

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