Title: N-terminal region is required for functions of the HAM family member

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Abstract
Shoot meristems contain stem cells and they sustain growth and development of above-ground tissues in land plants. The *HAIRY MERISTEM* (*HAM*) family genes, encoding GRAS-domain transcriptional regulators, play essential roles in control of shoot meristem development and stem cell homeostasis in several flowering plants. Similar to other GRAS proteins, the C-terminal regions of HAM family proteins across land plants are conserved, containing signature motifs that define the GRAS domain. In contrast, the N-terminal regions of HAM family proteins display substantial divergence in sequence and length. Whether the variable and divergent N-termini are required for the conserved functions of HAM proteins is unknown. Our recent work showed that *CrHAM*—the *HAM* homolog in the fern *Ceratopteris richardii* was able to replace the role of type-II *HAM* genes in Arabidopsis, maintaining established shoot apical meristems and promoting the initiation of new stem cell niches. Here, we provide additional information and show that CrHAM contains a much longer N-terminal region compared to Arabidopsis HAM proteins, which is conserved among different fern HAM homologs. The deletion of this region largely compromises the ability of CrHAM to replace the function of Arabidopsis HAM proteins in shoot meristems. These new data together with previous results suggest that, although lacking the sequence conservation among HAM homologs from different plant lineages, the N-termini are important for the conserved functions of HAM family proteins.

Key words:
GRAS family, HAM, Arabidopsis, shoot meristem, Ceratopteris

Main text
In land plants, shoot meristems sustain organogenesis and shape shoot architecture,[1, 2] and the GRAS domain HAIRY MERISTEM (HAM) family members play essential roles in control of the initiation and maintenance of shoot meristems in several dicot plants.[3-9] In the monocot plant maize, one *HAM* family member is preferentially expressed in the core region of shoot apical meristem (SAM) and is also involved in SAM development[10]. Our recent work further dissected the evolution history of the HAM family and showed the conserved regulatory mechanism underlying HAM expression and the conserved functions of the HAM family

members in land plants.[11] The C-terminal regions of HAM family proteins are conserved and contain five signature motifs: LHR I, VHIID, LHR II, PFYRE and SAW,[11, 12] as do other GRAS-domain proteins.[13, 14] In contrast to the conserved C-terminal regions, the N-terminal regions of HAM family proteins are highly variable in length and sequence, and no conserved domain or motif has been identified in these regions.[7, 11] Whether these variable N-terminal regions are required for functions of HAM family proteins is largely unknown.

To address this question, we further examined the *CrHAM* gene from the fern *Ceratopteris richardii* in this study, because ferns are sister to seed plants, sharing derived traits such as vascular systems and true leaves, and ferns also develop independent gametophytes similar to other seed-free plant lineages.[15] In addition, our recent work showed that the *pAtHAM2::YPET-CrHAM* expression cassette rescued the Arabidopsis *ham123* mutant,[11] demonstrating that CrHAM was able to replace the function of HAM1-3 in Arabidopsis SAMs. Interestingly, based on the alignment result,[11] we found that CrHAM had a much longer N-terminal sequence compared to Arabidopsis HAM proteins (Fig. S1). This N-terminal region contained the conserved amino acid residues that were shared among the HAM homologs from three fern species examined (*C. richardii*, *P. vittata*, and *S. cucullata*) (Fig. 1 and Fig. S2).

We generated a truncated *CrHAM* (hereafter referred as *CrHAM-C*), lacking 1131 base pairs that encoded the first 377 amino acid residues from the N-terminal region but maintaining the conserved microRNA 171 (miR171) binding site (5'-GATATTGGCGCGGCTCAATCA-3') (Fig. 2A and Notes S1). We then used the similar strategy described previously [11, 16] to explore the function and regulation of *CrHAM-C* in Arabidopsis. Specifically, we generated a *pAtHAM2::YPET-CrHAM-C* reporter, in which the *YPET-CrHAM-C* was driven by the promoter and 3' terminator from Arabidopsis *HAM2*. We then transformed this new reporter into the Arabidopsis *ham123* mutant. Through the confocal imaging, we found that the *pAtHAM2::YPET-CrHAM-C* reporter showed a concentration gradient from the epidermis to the deep cell layers in the SAMs (Fig. 2B-D), which was similar to previously reported expression patterns of *pAtHAM2::YPET-AtHAM2* [11, 16, 17] and *pAtHAM2::YPET-CrHAM* [11]. This result was consistent with our previous finding that the miR171 binding site played a conserved role in shaping the HAM expression pattern,[11] and it also indicated that the truncation of *CrHAM* did not affect its expression pattern in Arabidopsis SAMs.

We then characterized the phenotypes of the transgenic plants and we found that *pAtHAM2::YPET-CrHAM-C* failed to fully rescue the meristem defects in the *ham123* mutant (Fig. 3A-C and Fig. 4A-B). The *CLV3* expression pattern in the *pAtHAM2::YPET-CrHAM-C* in *ham123* plant (Fig. 3C) was different from that in wild type (Fig. 3A). The ectopic expression of *CLV3* in the rib meristem of the *pAtHAM2::YPET-CrHAM-C in ham123* SAM (Fig. 3C) was similar to that previously observed in the *ham123* mutant (Fig. 3B) [5, 8, 11, 16]. Moreover, compared to the wild type control (Fig. 4A), the *pAtHAM2::YPET-CrHAM-C* in *ham123* plants showed reduced shoot branching and the defects in the initiation of new stem cell niches (Fig. 4B). Thus, the deletion of N-terminus largely compromised the ability of CrHAM to fully rescue the *ham123* mutant phenotype. These analyses suggested that

although lacking the sequence conservation among HAM homologs from different plant lineages, N-terminal regions are important for functions of HAM family members in control of stem cell homeostasis.

Materials and Methods

Sequences of the HAM homologs from *Ceratopteris richardii* (CrHAM), *Pteris vittata* (PvHAM), *Salvinia cucullata* (ScHAM), Arabidopsis thaliana (AtHAM1-4), *Pinus pinaster* (PpiHAM), and *Physcomitrella patens* (PpHAM) were included in our recent study.[11] Sequence alignment was performed using MAFFT with E-INS-I and default settings.[18] The Arabidopsis *ham123* (*ham1ham2ham3*) mutant was previously described.[5,7] The *CrHAM-C* coding sequence was amplified from the cDNAs of *C. richardii* wild-type strain Hn-n[19] with the primers 5'-ATGTCGCCTTCCATCCAGGTA-3' and 5'-TCAAGGTCCCCATGCAGATG - 3'. The *pAtHAM2::YPET-CrHAM-C* expression cassette was generated using the method described,[11] including the same promoter and 3' untranslated sequence of Arabidopsis *HAM2* used in the *pAtHAM2::YPET-AtHAM2* and *pAtHAM2::YPET-CrHAM* reporters.[11] The *pAtHAM2::YPET-CrHAM-C* was introduced into the *pMOA34* binary vector,[20] and the *pMOA34 pAtHAM2::YPET-CrHAM-C* construct was transformed into the *ham123* mutant through the floral dip method.[21] More than three independent transgenic lines have been identified, which showed the comparable expression patterns in the SAMs and comparable growth phenotypes. The confocal imaging of *pAtHAM2::YPET-CrHAM-C* was performed as previously described[5, 11, 16, 17, 22]. For RNA *in situ* hybridization, plants were grown in short days at 22°C and harvested at 27 days after germination, the same condition described in the recent study[11]. RNA *in situ* hybridization in the SAMs from different genotypes was performed using the identical procedures described[11], and at least three biological replicates showed similar results. For the observation of axillary shoot development, plants were initially grown under the same short-day conditions and then transferred to continuous light at 22°C as described[11]. The expression pattern and phenotypic characterization of the full-length *CrHAM* transgenic line (*pAtHAM2::YPET-CrHAM in ham123*) were published[11]. The image showing the *ham123* mutant grown in the same condition (shown in Figure 4) was published recently[11]. Schematic diagrams of protein domain structures were generated using DOG 2.0.[23] The conserved motifs (LHR I, VHIID, LHR II, PFYRE and SAW) were defined based on the previous studies.[11-12]

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Author contributions

YG and YZ conceived the research direction, YG performed the experiments, YZ supervised the research activities and discussed the experimental results, and YG and YZ wrote the manuscript.

Figure legends

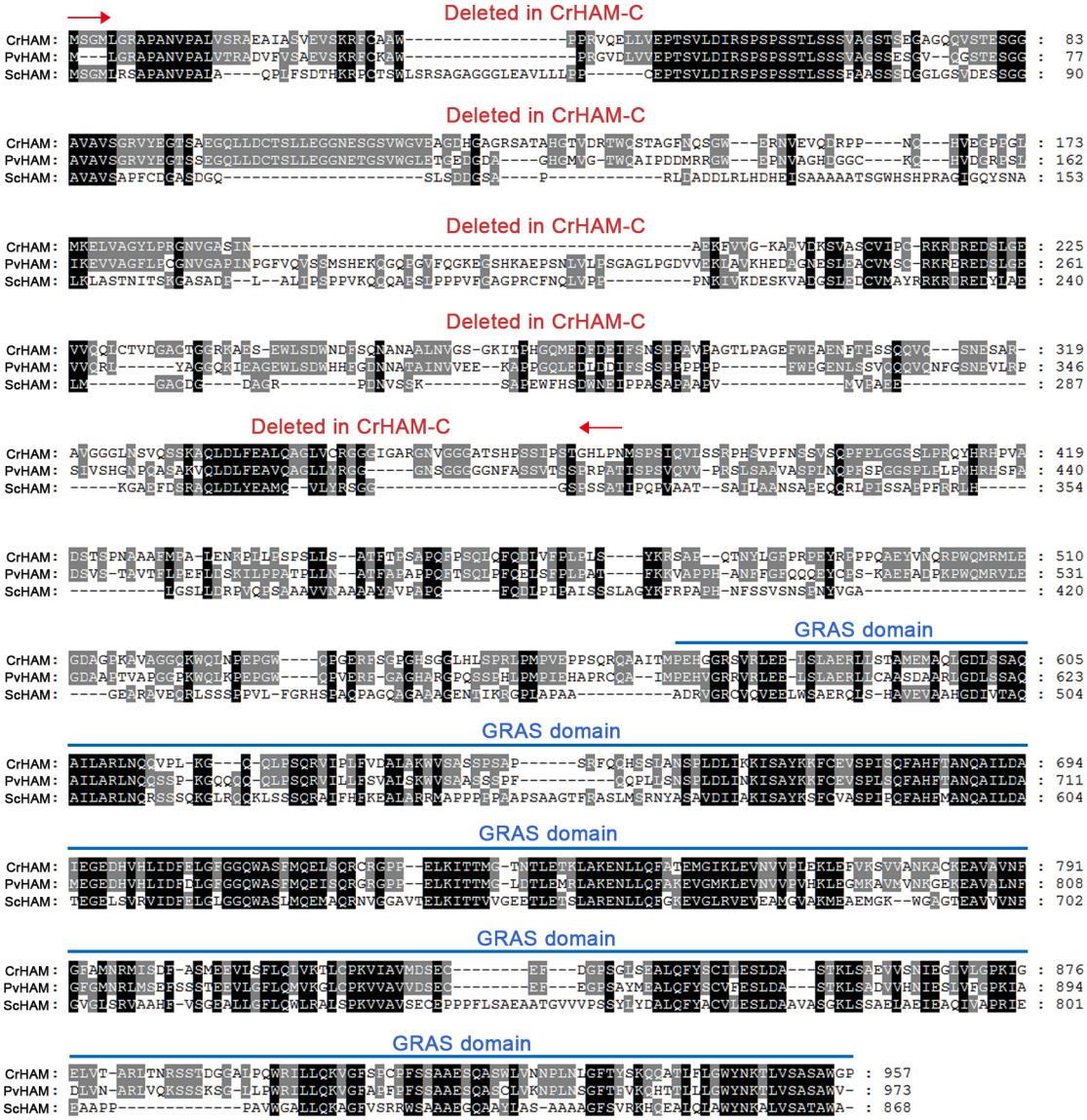

Figure 1. Alignment of HAM homologs from three fern species. Cr: *Ceratopteris richardii*; Pv: *Pteris vittata*; Sc: *Salvinia cucullata*.

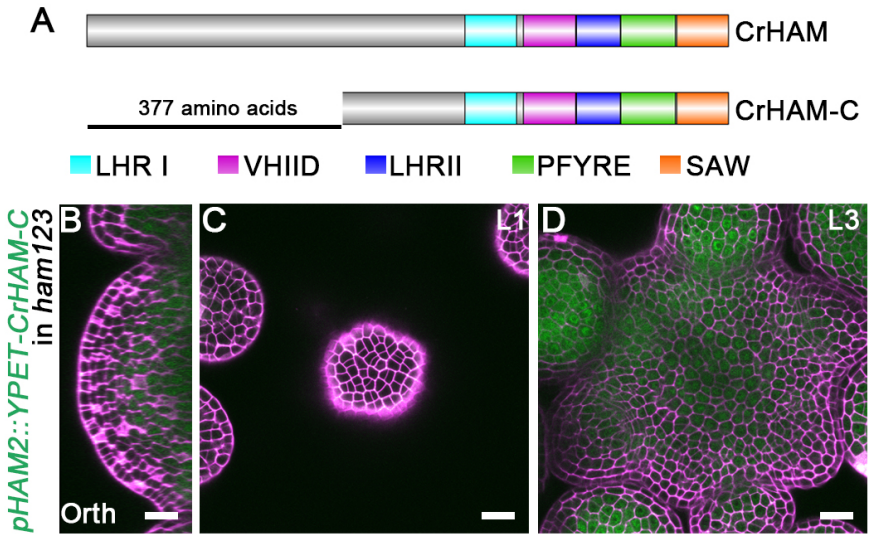

Figure 2. The expression of a truncated CrHAM protein (CrHAM-C) in Arabidopsis shoot apical meristems. (A) Schematic diagram showing the full-length CrHAM protein and the truncated CrHAM protein lacking the N-terminal 377 amino acids (CrHAM-C). (B-D) Confocal imaging of *pHAM2::YPET-CrHAM-C* in the SAM of the *ham123* mutant, from orthogonal view (B), and transverse section views in L1 (C) and corpus (D). Green: YPET-CrHAM-C; Purple: PI (Propidium iodide) stain. Scale bars: 20 μm.

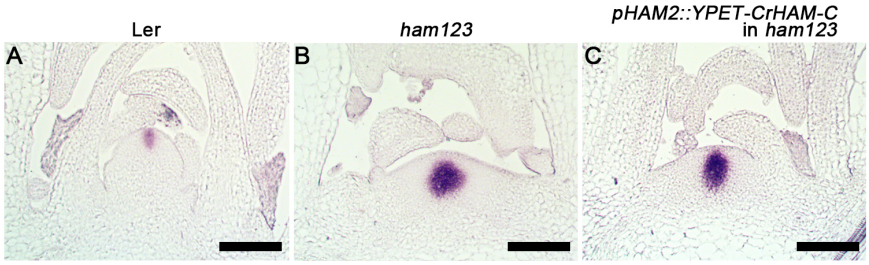

Figure 3. Functional analysis of *CrHAM-C* in Arabidopsis SAMs. (A-C) RNA *in situ* hybridization to *CLV3* mRNAs in the SAMs of Arabidopsis wild type L*er* (A), *ham123* mutant (B), and the *ham123* mutant expressing *pHAM2::YPET-CrHAM-C* (C). Scale bars: 100 μm.

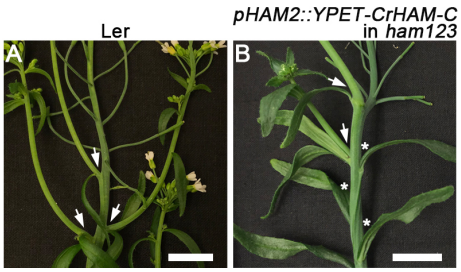

Figure 4. Functional analysis of *CrHAM-C* during Arabidopsis axillary shoot development. (A-B) Images of branches initiated from the base of the cauline leaves in the Arabidopsis L*er* wild type (A) and the *ham123* mutant expressing *pHAM2::YPET-CrHAM-C* (B). Arrows indicate the branches that are normally initiated from the base of the cauline

leaves. Asterisks indicate the absence of branches initiated from the base of cauline leaves. The plants (A-B) were grown in the same condition. Scale bar: 1 cm.

Figure S1. Schematic diagram showing the full-length CrHAM protein and Arabidopsis HAM proteins.

Figure S2. Alignment of HAM homologs from different land plant species. Cr: *Ceratopteris richardii*; Pv: *Pteris vittata*; Sc: *Salvinia cucullata*; At: *Arabidopsis thaliana*; Ppi: *Pinus pinaster*; Pp: *Physcomitrella patens*.

Notes S1. **Nucleotide sequences of *CrHAM* and *CrHAM-C***.

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