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CaHAM is autoregulated and regulates *CaSTM* expression and is required for shoot apical meristem organization in pepper

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ABSTRACT

The angiosperm shoot apical meristem (SAM) is characterized by tightly organized cell layers and zones. The SAM's organization allows it to maintain its indeterminate nature while producing determinate lateral organs. Alterations in SAM gene expression partly account for the immense diversity in plant architecture. The GRAS protein family gene *HAIRY MERISTEM* (*HAM*) is an important regulator of SAM organization in *Petunia* and *Arabidopsis*. Here we describe *CaHAM* loss-of-function pepper mutants characterized by an arrested SAM following the formation of several leaves on the primary stem, complete inhibition of axillary meristem development, an expanded tunica domain and trichome formation on the SAM epidermis. *CaHAM* is expressed in the periphery of the SAM and in the vasculature of young leaves throughout plant development, reaching its highest level in the reproductive growth stage. Analysis of the effect of *CaHAM* loss-of-function on its own expression showed that *CaHAM* is negatively autoregulated. Furthermore, *CaHAM* negatively regulates the expression level and pattern of pepper *SHOOT MERISTEMLESS* (*CaSTM*), which is required to maintain the SAM in an undifferentiated state. We conclude that *CaHAM* is regulated to achieve adjusted functional levels and has a conserved role in controlling SAM maintenance, organization and axillary meristem formation.

1. Introduction

The shoot apical meristem (SAM) plays a fundamental role in stem formation and in continuous lateral organ production in angiosperms [1]. To stay active, the SAM must generate and maintain new meristematic cells in its center and allow cell differentiation at its periphery. The SAM is characterized by radial zonation, including the central zone where meristematic cells are generated, the peripheral zone where lateral organs appear, and the rib meristem which gives rise to the stem tissue. The SAM is organized into a tunica (the superficial L1 and L2 layers), where cell division occurs in the anticlinal plane, and a corpus (the interior of the meristem), where cell division occurs in all planes [1]. This precise organization results from tight developmental regulation governed by key genes, including the Class I *KNOTTED1*-like homeobox gene *SHOOT MERISTEMLESS* (*STM*) which encodes a homeodomain transcription factor and is required for both SAM initiation and its maintenance in the undifferentiated state [2]. *STM* is a marker of meristem identity as it is expressed throughout the SAM and is absent from differentiated leaf founder cells [3,4].

The size of the SAM is mainly regulated by the feedback network between *CLAVATA* (*CLV*) and *WUSCHEL* (*WUS*) genes [5]. *WUS* encodes a homeodomain transcription factor that is expressed beneath the meristem stem cell niche and acts to both initiate and maintain the stem cells in the undifferentiated state [6]. *CLV3* encodes a peptide ligand that is expressed predominantly in the tunica and binds to the CLV1/CLV2 receptor complex, which is mainly expressed in the corpus [7–11]. WUS activates *CLV3* expression; CLV3 binds to the CLV1/CLV2 complex and together they suppress *WUS* expression [12,13]. Such regulation ensures correct development of the indeterminate shoot apex.

Indeterminacy of the SAM is maintained by several factors, including *HAIRY MERISTEM* (*HAM*), which is a transcription factor of the GRAS protein family first identified in *Petunia* [14]. It has been suggested that *HAM* promotes shoot indeterminacy via non-cell-autonomous signaling, as it is expressed in the peripheral zone of the SAM while its expression is excluded from the central zone [14]. *ham* loss-of-function mutants exhibit premature cessation of the shoot apex, epidermis differentiation on the SAM, and arrest of axillary shoot development [14]. It has been suggested that *HAM* affects cells in their response to *WUS* and *STM*. Three recent studies have identified *AtHAM*/*LOM*/*SCL6* as the *Arabidopsis HAM* homologs [15–17]. *Arabidopsis* possesses three *HAM* homologs, of which *AtHAM1* and *AtHAM2* act redundantly to promote *HAM* function in the peripheral zone of the SAM and in axillary

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meristem development, whereas *AtHAM3* functions primarily in axillary meristem development, redundantly with *AtHAM1* [15,16]. *HAM* genes possess a microRNA-binding site and post-transcriptional regulation contributes to their role in plant development [17].

Mutant analysis in different plant species has revealed that axillary meristem initiation is controlled by a number of genes. These include *LATERAL SUPPRESSOR* (*LAS*), a member of the GRAS protein family that controls axillary meristem formation in tomato and *Arabidopsis* [18,19]; *REVOLUTA* (*REV*), which regulates both apical and axillary meristem formation in *Arabidopsis* [20,21]; *BLIND* (*BL*), which is required for axillary meristem initiation in tomato, *Arabidopsis* and pepper [22–24], and *REGULATOR OF AXILLARY MERISTEM FORMATION* (*ROX*), which was recently characterized in *Arabidopsis* [25]. *ROX* is the ortholog of the branching regulators *LAX PANICLE1* (*LAX1*) in rice and *barren stalk1* (*ba1*) in maize.

While genes and pathways controlling plant architecture have been identified in a limited number of model species, additional species exhibiting variable shoot architectures need to be studied to determine the extent of gene-function conservation in these plants. Pepper (*Capsicum annuum*) is an important vegetable crop and a member of the Solanaceae family with a sympodial growth habit. Pepper exhibits a bifurcated shoot in which two sympodial units are initiated at the axils of the two opposite leaves subtending the single terminal flower [26,27]. To unravel the genetic control of pepper shoot architecture, we generated EMS (ethyl methanesulfonate)-induced mutants and searched for plants that are altered in their shoot branching, sympodial shoot development and flower development [28]. We then identified genes controlling shoot meristem activity, including *CaANANTA*, *CaBLIND* and *CaJOINTLESS* [26,29,30]. In the current report, we describe the isolation of loss-of-function pepper mutants that exhibit SAM termination and arrested axillary shoot production. We show that this phenotype is controlled by a mutated *CaHAM* gene, the pepper homolog of *HAM*, and describe its role in the organization and maintenance of the pepper SAM.

2. Materials and methods

2.1. Plant material

Pepper accessions of *C. annuum* were derived from an EMS mutant population of the blocky cultivar cv. Maor [28]. The E576, E1402 and E394 alleles were isolated from two independent screenings of 3000 M2 families for plants with altered plant architecture. Line numbers represent family serial number. Allelism tests were performed by crossing the three mutants and analyzing the phenotype of their F1 progenies. The mutants were backcrossed to the wild type before further genetic analysis. An F2 mapping population was generated by crossing E576 to *C. frutescens* BG 2816.

2.2. Microscopy

For scanning electron microscopy (SEM) analysis, pepper apices were fixed in FAA (formaldehyde:acetic acid:70% ethanol, 10:5:85) as described previously [31]. Electronic images were obtained with a Jeol JSM-5410 LV scanning electron microscope (Japan). For histological analysis, shoot apices were fixed in FAA, dehydrated through an ethanol series (70, 80, 90, and 100%, 30 min each), embedded in paraffin, sectioned in a microtome (Leica RM2245, Germany), and stained with Safranin-O/Fast-green [32]. The sectioned material was observed in a Leica IM1000 microscope, and digital images were taken with a CCD camera DC2000.

2.3. Phylogenetic analysis

The phylogenetic tree was calculated using the neighbor-joining method and bootstrap analysis with 1000 replicates using MEGA4 software [33]. The tree was calculated from alignments of the amino acids of the conserved GRAS domain of HAM homologs in pepper, petunia, tomato and *Arabidopsis*.

2.4. Real-time RT-PCR

Total RNA was extracted from shoot apices using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma, USA) according to the manufacturer's instructions. Following DNaseI digestion (Sigma), 0.5 μg of total RNA from three biological replicates was reverse-transcribed with Reverse-iT™ 1st Strand Synthesis Kit and oligo-dT primer according to the manufacturer's protocol (Thermo Scientific ABgene, UK).

Real-time RT-PCR analysis was performed using ABsolute Blue SYBR Green ROX Mix (Thermo Scientific ABgene) in a Rotor-Gene 6000™ (Corbett Life Science, Australia). cDNA was diluted seven times before being subjected to quantitative RT-PCR analysis. Three biological replicates were used for each developmental stage, and each cDNA sample was run in triplicate. Gene-product specificity was confirmed by melting curve analysis and a negative control without cDNA was also run. Specific gene expression was normalized to the internal control gene *UBIQUITIN* using primers 5′-GGTCCTGTTGTCCATTTGCT-3′ and 5′-GTCTCGTATTTGGCCCTGTC-3′. The gene-expression value of the wild type was used as a control and set to 1.0. The primers that were used for expression analysis included:

CaHAM F 5′-GCCATTAACTGTGGGGTCGG-3′ and R 5′-CTCCACATCACCTGAAATCCACC-3′
CaBL F 5′-GGTGTTTTTGGGGTTTTGTGTAGG-3′ and R 5′-GAATCAAAATCTCAAAACGAATATCAAACATC-3′
CaSTM F 5′-GAAGATATGCAGTTTGTGGTGATG-3′ and R 5′-CTCCACAATATTCAGAGGAGAGATG-3′
CaREV F 5′-TGGATTCAGTGACGATGGCTG-3′ and R 5′-GAGGAACATTCTGGAGTAGCATTG-3′
CaLAS F 5′-TTTCTTCCATTGTTCTCCTCCCTG-3′ and R 5′-AACCCTATGCAAGAAAACCCTTAGTT-3′.

2.5. In situ hybridization

In situ analysis with digoxigenin (DIG)-labeling was performed as previously described [34,35]. Shoot apices were fixed in FAA, dehydrated and embedded in ParaPlast (McCormick Scientific, USA). The tissue was then sectioned (10 μm) on a Leica RM2245 microtome, mounted in VectaMount (Vector Laboratories, USA) and sections were placed on SuperFrost Plus slides (Menzel-Glaser, Germany) for 2 days on a 42 °C hot plate. An antisense DIG-labeled RNA probe was synthesized from a PCR product that was amplified from *CaHAM* cDNA, using forward primer 5′-GGGGTTCTTTGTGTTTCTAATTTTGTG-3′ and reverse primer 5′-CTAATACGACTCACTATAGGGACCCCACAGTGC-3′ which possessed a T7 RNA polymerase promoter sequence. The *CaSTM* template was similarly amplified using forward primer 5′-GGATTGGACCAGAAGCAAATAAACAACTGG-3′ and reverse primer 5′-CTAATACGACTCACTATAGGGGAAATGGTTAGC-3′. The RNA probes were synthesized using the MEGAscript kit (Ambion, USA) and DIG RNA-labeling mix (Roche Applied Science, USA). The probes were later purified using the MEGAclear kit (Ambion), and quantified by running 1 μl on an agarose gel. Analyzed sections were mounted in VectaMount and recorded under a microscope (DMLB, Leica) using a digital camera (NikonDs-Fs-fi1).