RESEARCH PAPER

**HANABA TARANU** regulates the shoot apical meristem and leaf development in cucumber (Cucumis sativus L.)

Lian Ding¹, Shuangshuang Yan¹, Li Jiang¹, Meiling Liu¹, Juan Zhang¹, Jianyu Zhao¹, Wensheng Zhao¹, Ying-yan Han², Qian Wang¹ and Xiaolan Zhang¹,*

¹ Department of Vegetable Sciences, Beijing Key Laboratory of Growth and Developmental Regulation for Protected Vegetable Crops, China Agricultural University, Beijing 100193, China
² Department of Plant Science and Technology, Beijing University of Agriculture, Beijing, 102206, China

* To whom correspondence should be addressed. E-mail: zhxiaolan@cau.edu.cn

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Abstract

The shoot apical meristem (SAM) is essential for continuous organogenesis in higher plants, while the leaf is the primary source organ and the leaf shape directly affects the efficiency of photosynthesis. **HANABA TARANU (HAN)** encodes a GATA3-type transcription factor that functions in floral organ development, SAM organization, and embryo development in Arabidopsis, but is involved in suppressing bract outgrowth and promoting branching in grass species. Here the function of the **HAN** homologue **CsHAN1** was characterized in cucumber, an important vegetable with great agricultural and economic value. **CsHAN1** is predominantly expressed at the junction of the SAM and the stem, and can partially rescue the **han-2** floral organ phenotype in Arabidopsis. Overexpression and RNAi of **CsHAN1** transgenic cucumber resulted in retarded growth early after embryogenesis and produced highly lobed leaves. Further, it was found that **CsHAN1** may regulate SAM development through regulating the **WUSCHEL (WUS)** and **SHOOT MERISTEMLESS (STM)** pathways, and mediate leaf development through a complicated gene regulatory network in cucumber.

Key words: **CsHAN**, **CsSTM**, **CsWUS**, cucumber, leaf development, shoot apical meristem.

Introduction

The shoot apical meristem (SAM) is crucial for continuous organogenesis in higher plants. All the aerial organs including leaves, flowers, and stems are initiated from the SAM. The SAM is generally established during embryogenesis with a dome-shaped morphology, and can be divided into three functional zones: (i) the central zone with self-maintaining stem cells at the centre of the SAM; (ii) the peripheral zone where the lateral organ primordia are initiated from the shoulder of the SAM; and (iii) the rib zone in which stem tissue is specified beneath the central zone of the SAM (Steeves and Sussex, 1989; Fletcher, 2002; Tucker and Laux, 2007). Two independent pathways have been identified to be required for meristem establishment and maintenance in Arabidopsis, one is the **WUSCHEL (WUS)**–**CLAVATA (CLV)** pathway...
(Brand et al., 2000; Schoof et al., 2000). WUS, a homeodomain transcription factor, is expressed in the centre of the SAM, called the organizing centre, and functions to promote meristematic cell fate (Mayer et al., 1998). Mutation in WUS leads to a premature SAM with no ability to self-maintain the stem cells (Laux et al., 1996). CLV3, a signalling peptide, directly binds to the plasma membrane-localized receptor-like kinases CLV1 or CLV2/CRN complex and transmits a signal that restricts WUS expression, while WUS promotes the expression of CLV3 in the stem cells as a feedback loop (Fiers et al., 2005; Ito et al., 2006; Kondo et al., 2006; Ogawa et al., 2008; Bleckmann et al., 2010; Yadav et al., 2011). SHOOT MERISTEMLESS (STM) is the other pathway that is essential for meristem maintenance. STM, a KNOTTED1-LIKE HOMEobox (KNOX) gene, is expressed throughout the SAM but is excluded from the organ primordia that function to maintain the undifferentiated cells in the SAM (Endrizzi et al., 1996; Long et al., 1996; Lenhard et al., 2002). KNAT1/ BREVIPEDICELLUS (BP), another member of the KNOX family, plays a role in meristem maintenance partially redundant with STM (Byrne et al., 2002; Douglas et al., 2002).

Leaf is the primary source organ, and the leaf shape directly affects the efficiency of photosynthesis (Tsukaya, 2006; Nicotra et al., 2008). The leaf primordium is initiated from the peripheral zone of the SAM, in which STM is down-regulated (Long et al., 1996) and ASYMMETRIC LEAVES 1 and 2 (AS1/2) are up-regulated (Ori et al., 2000; Guo et al., 2008). Leaves of as1 and as2 mutants are downward curling with asymmetric lobes and short petioles (Byrne et al., 2000; Iwakawa et al., 2002; Iwakawa et al., 2007). AS1 and AS2 form a protein complex that directly represses BP and KNAT2 transcription (Guo et al., 2008). Consistently, ectopic expression of KNOX genes results in lobed leaves in simple leaf species, and super-compoundness in compound leaf species such as tomato (Lincoln et al., 1994; Chuck et al., 1996; Janssen et al., 1998; Hake et al., 2004; Belles-Boix et al., 2006). Further functional studies assessed a key role for KNOX genes in leaf shape determination (Hay and Tsiantis, 2010; Di Giacomo et al., 2013; Bar and Ori, 2015). Several additional regulators have been found to mediate leaf shape development. For example, mutation in SERRATE (SE), a zinc finger protein involving in a miRNA gene silencing pathway, results in serrated leaves in Arabidopsis (Prigge and Wagner, 2001). Cap Binding Protein 20 (CBP20) encodes the 20kDa subunit of the nuclear mRNA cap-binding complex (nCBC), and a cbp20 mutant shows a serrated leaf margin (Papp et al., 2004). Mutation of ARGONAUTE1 (AGO1), a key player in transgene-induced post-transcriptional gene silencing, also leads to serrated leaves (Bohmer et al., 1998; Morel et al., 2002). AGO10/PINHEAD (PHN), another AGO protein gene, represses the accumulation of miR165/166, thereby affecting the establishment of leaf polarity (Liu et al., 2009). JAGGED (JAG), a C2H2-type zinc finger transcription factor gene, is expressed in the initiating lateral organ primordia and is essential for proper leaf shape. jag mutants show narrow and serrated leaves, and the gain-of-function mutant jag-5D has bract-like organs subtending most flowers (Dinneny et al., 2004; Ohno et al., 2004). Boundary genes CUP-SHAPED COTYLEDON (CUCI, 2 and 3) were initially identified by defective SAM development and organ fusion (Aida et al., 1999; Aida and Tasaka, 2006). Recently, CUC genes have been shown to play an important role in leaf margin development in both simple and compound leaf species and to act downstream of KNOX transcription factor genes (Nikovics et al., 2006; Blein et al., 2008; Berger et al., 2009; Bilsborough et al., 2011; Hasson et al., 2011; Spinelli et al., 2011). In Arabidopsis, genetic interactions among these different regulators lead to increased dissection of the Arabidopsis leaf margins (Blein et al., 2013).

HANABA TARANU (HAN) is a boundary gene that regulates SAM organization and flower organ development in Arabidopsis (Zhao et al., 2004). HAN encodes a GATA3 transcription factor that is expressed in the boundaries between the meristem and developing organ primordia, the boundaries between different floral whors, as well as the junctional domain between the SAM and the stem (Zhao et al., 2004). Mutation of HAN leads to fused sepals, reduced floral organs, and a flatter SAM (Zhao et al., 2004). Previous studies showed that HAN and three GATA3 family genes, HAN2 (HAN-LIKE 2), GNC (GATA, NITRATE-INDUCIBLE, CARBON-METABOLISM-INVOLVED), and GNL (GNC-LIKE), form a negative feedback loop to regulate flower development (Zhang et al., 2013). The functions of HAN homologues are divergent in different species. For example, HAN homologues such as TASSEL SHEATH1 (TSH1) in maize (Zea mays), NECKLEAF1 (NLI) in rice (Oryza sativa), and THIRD OUTER GLUME (TRD) in barley (Hordeum vulgare) are involved in repressing bract outgrowth and promoting branching (Wang et al., 2009; Whipple et al., 2010).

In this study, the function of HAN was explored in cucumber (Cucumis sativus L.), a globally cultivated vegetable that is of important economic and nutritional value (Huang et al., 2009). Unlike the model plant Arabidopsis and most crops, cucumber is a typical unisexual plant with indeterminate growth, continuously producing leaves and male or female flowers simultaneously (Málepszy and Niemirowicz-Szczytt, 1991; Kater et al., 2001; Hao et al., 2003; Bai et al., 2004). Two HAN homologous genes were identified in cucumber, and the function of CsHAN1 was characterized in detail. CsHAN1 is predominantly expressed at the junction of the SAM and the stem, and can partially rescue the han-2 floral organ phenotype in Arabidopsis. Overexpression or down-regulation of CsHAN1 in the transgenic cucumber plants led to retarded growth and lobed leaves. Further, it was found that CsHAN1 may regulate SAM development through bridging the WUS and STM pathways, and mediate leaf margin development through a complicated gene regulatory network in cucumber.

**Materials and methods**

**Plant materials and growth conditions**

Cucumber (Cucumis sativus L.) inbred line R1407, which is a northern China type cucumber with dark green fruits similar to the sequenced line 9930, was used in this study. The cucumber seedlings were grown in a growth chamber under 16h/8h and 25 °C/18 °C day/night until the two true-leaf stage, and the cucumber plants...
were then transferred to a greenhouse in the experimental field of China Agricultural University in Beijing. Pest control and water management were carried out according to standard protocols. The *Arabidopsis thaliana* Landsberg erecta (Ler) and Columbia (Col) ecotypes, and the mutant alleles *han-2* (*Ler*) were described previously (Zhao et al., 2004; Zhang et al., 2013) and obtained from the Meyerowitz lab stock collection. The mutant allele *han-2* (*Col*) was obtained by crossing *han-2* (*Col*) to Col followed by six generations of selfing. The *Arabidopsis* plants were grown in a growth chamber under 16 h light/8 h dark at 22 °C.

**Gene cloning and phylogenetic analysis**

Total RNA was extracted from the cucumber floral buds using a Quick RNA isolation Kit (Waryoung, China), and cDNA was synthesized using a Promega reverse transcriptase kit (Promega, USA). The coding sequences (CDS) of *CsHAN1* and *CsHAN2* were obtained using gene-specific primers (Supplementary Table S1 available at JXB online). The gene structure analysis was performed using online software GSDDS 2.0 ([http://gsds.cbi.pku.edu.cn/](http://gsds.cbi.pku.edu.cn/)). The amino acid sequences of related HAN proteins in other species were obtained by BLAST searches ([http://www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)). Protein alignment of CsHAN and related HANs was performed using ClustalW in the MEGA5 software package, and the boxes were drawn using the BoxShade web site ([http://www.ch.embnet.org/software/BOX_form.html](http://www.ch.embnet.org/software/BOX_form.html)). The phylogenetic analysis based on amino acid sequences was performed using the Neighbor–Joining (NJ) method with 1000 bootstrap replicates through MEGA5 software (Saitou and Nei, 1987).

**Quantitative real-time PCR**

Total RNA was extracted from different cucumber tissues or *Arabidopsis* inflorescences using a Quick RNA isolation Kit (Waryoung, China), and cDNA was synthesized using a Promega reverse transcriptase kit (Promega, USA). An ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, USA) was used for quantitative real-time reverse transcription–PCR (qRT–PCR) experiments. Three biological and three technical replicates (3 × 3) were performed for each gene. The cucumber *Ubiquitin extension protein (UBI-ep)* gene and the *Arabidopsis ACTIN2* gene were used as internal references to normalize the expression data. The standard deviation was calculated between three biological replicates, using the average of the three technical replicates for each biological sample. The gene-specific primers are listed in Supplementary Table S1 at JXB online.

**In situ hybridization**

Cucumber shoot apices of 6-, 12-, and 15-day-old seedlings, male and female buds, and young fruits from 0.8 cm to 2.8 cm were fixed in 3.7% formalin–acetic acid–alcohol (FAA), and *in situ* hybridization was performed as described previously (Zhang et al., 2013). Sense and antisense probes were synthesized by PCR amplification using SP6 and T7 RNA polymerase, respectively. Probes of *CsWUS*, *CsSTM*, and *CsBP* were designed according to the specific gene sequences. The primers for probe generation are listed in Supplementary Table S1 at JXB online.

**Ectopic expression of CsHAN1 in Arabidopsis**

To make the *CsHAN1* overexpression construct, the full-length *CsHAN1* CDS were amplified and cloned into the binary vector pBI121 through *XbaI* and *SalI* sites. The recombinant plasmids were introduced into *Agrobacterium* by electroporation and then transformed into wild-type (WT) and *han-2* mutant plants through the floral dip method (Clough and Bent, 1998). The transgenic plants were screened on Murashige and Skoog (MS) medium with 40 mg l⁻¹ kanamycin. The primers for vector construction are listed in Supplementary Table S1 at JXB online.

**Cucumber transformation**

The same *CsHAN1* overexpression construct was used for cucumber transformation. To generate *CsHAN1-RNAi* transgenic plants, the 258 bp sense and antisense fragments from the 3' end of *CsHAN1* were amplified using gene-specific primers containing SpeI (5' end)/ScaI (3' end) and BamHI (5' end)/KpnI (3' end) sites, respectively. The two fragments were inserted into the RNAi-1 vector, and the empty RNAi-1 vector was used as a transformation control. The resultant *CsHAN1-RNAi* construct and empty RNAi-1 vector were then delivered into *Agrobacterium* by electroporation and transformed into the cucumber inbred line R1407 line using the cotyledon transformation method as previously described (Wang et al., 2014). The primers containing the restriction enzyme cutting sites are listed in Supplementary Table S1 at JXB online.

**Paraffin sections**

Young cucumber seeds at 16 d after fertilization were fixed, embedded, sectioned, and dewaxed as described (Jiang et al., 2014). Sections of 8 μm thickness were mounted in neutral resins, and images were taken under a light microscope (D72, Olympus, Japan).

**Accession numbers**

Sequence data in this paper can be found in the Cucumber Genome DataBase, TAIR, or GenBank under the following accession numbers: *CsHAN1* (Csa016191), *CsHAN2* (Csa012029), *CsPNH1* (Csa015921), *CsPNH2* (Csa004392), *CsAGO1* (Csa000946), *CsJAG* (Csa008074), *CsAS2* (Csa012250), *CsBP* (Csa009344), *CsKNT2* (Csa013896), *CsKNT* (Csa011388), *CsWUS* (Csa00479), *CsSTM* (Csa000554), *AtHAN* (AT3G50870), *SE* (AT2G27100), *AGO1* (AT1G48410), *AS2* (AT1G65620), *KNT2* (AT1G70510), *CPB20* (AT5G4200), *BP* (AT4G08150), *CUC3* (AT1G76420), *PNH* (AT5G43810), *JAG* (AT1G68480), *GNV* (AT5G56880), *GNL* (AT4G26150), *HvTRD* (GU722206), *OsNL1* (DQ784546), and *ZmTSH1* (AC199892.4_FG031).

**Results**

**Isolation of the cucumber *CsHAN* genes**

To identify the HAN homologues from cucumber, a BLAST search was performed in the Cucumber Genome DataBase (Huang et al., 2009) based on the amino acid sequence information of *Arabidopsis HAN*. Two candidate genes, Cs016191 and Cs012029, showed the highest similarity. A further BLAST search was performed in TAIR ([http://www.arabidopsis.org/](http://www.arabidopsis.org/)) using the two candidate gene, and both of them got the best hit to *Arabidopsis HAN* (AtHAN). Thus, Cs016191 was named *CsHAN1* and Cs012029 was named *CsHAN2*, respectively, and their CDS as well as their genomic sequence from the flower buds of cucumber line R1407 were cloned. Gene structure analysis showed that *CsHAN1* and *CsHAN2*, encoding 255 and 214 amino acids, respectively, contain two exons and one intron, consistent with the gene structure of *AtHAN* and *HAN* homologues (Zhao et al., 2004; Wang et al., 2009; Whipple et al., 2010) (Fig. 1A). Previous studies showed that *HAN* encodes a GATA3-like transcription factor with a single zinc finger domain and a HAN motif (Whipple et al., 2010). Protein alignment of HAN homologues from *Arabidopsis* (AtHAN), rice (OsNL1), maize (ZmTSH1), and cucumber (CsHAN1/2) was performed using ClustalW in the MEGA5 software. Despite *CsHAN1* and *CsHAN2* showing only 39.55% and 34.46% identity with AtHAN, respectively, the GATA zinc finger domain and the HAN motif are highly conserved (Fig. 1B).
Phylogenetic analysis of the deduced HAN proteins from various species was performed using the NJ method (Saitou and Nei, 1987). The phylogenetic tree showed that HAN homologues in the eudicot species form a distinct clade from those in the monocotyledon species such as rice, maize, and barley (Fig. 1C). In watermelon (Citrus lanatus), another Cucurbitaceae species, there are also two HAN homologues (Clal014581 and Clal014213) as well, and they formed two different clades with CsHAN1 and CsHAN2, respectively (Fig. 1C), implying that HAN homologues in Cucurbitaceae may have a distinct function from that in other species. (This figure is available in colour at JXB online.)
in the model *Arabidopsis* plant. Given that CsHAN1 is more closely related to AtHAN than CsHAN2 (Fig. 1C), CsHAN1 was chosen and analysed in this study.

**Expression pattern of CsHAN1 in cucumber**

The expression of *CsHAN1* was examined in different organs of cucumber through qRT–PCR (Fig. 2A). Total RNA was

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**Fig. 2.** Expression analysis of *CsHAN1* in cucumber. (A) Quantitative RT–PCR (qRT–PCR) analysis of *CsHAN1* in different tissues of cucumber. YL, young leaves; FB, female buds; MB, male buds; FF, female flowers; MF, male flowers; F-4, young fruits 4 d before anthesis; F, fruit at anthesis; F+3, fruits 3 d after anthesis. The *Ubiquitin extension protein* (*UBI-ep*) gene was used as an internal reference to normalize the expression data. (B–K) *In situ* hybridization with the *CsHAN1* antisense probe (B–J) and sense probe (K). (B) In the cucumber shoot apex, *CsHAN1* is expressed in the junction region of the inflorescence meristem (IM) and stem (arrow), the junction regions of the floral meristem (FM) and stem (asterisk), and the axil of leaf primordia (arrowhead). (C–E) Floral buds at stage 2 (C), 3 (D), and 4 (E). Asterisks show the expression domain of *CsHAN1* at the junction of the meristem and stem, and arrows indicate the expression of *CsHAN1* at the boundary between the petal and stamen, and the boundary between the stamen and initiating carpel primordia. (F–G) Male flowers at stage 9 (F) and stage 11 (G); (G’) is a high magnification view of the anther in (G). The signal of *CsHAN1* was detected in the developing anther, tapetum cell layer, and the uninuclear pollen. (H) Female flower in stage 8. *CsHAN1* is expressed in the ovary (arrow). (I, J) Cross-sections of the female ovary in stage 9 (I) and stage 10 (J) showing the expression domain of *CsHAN1* in the ovules and the base of the embryo sac. (K) No signal was found on hybridization with the sense *CsHAN1* probe. S, sepal; P, petal; St, stamen; C, carpel; Ov, ovule; In, integument; Es, embryo sac. Bar=100 μm. (This figure is available in colour at JXB online.)
extracted from young leaves, female flower buds, male flower buds, female opening flowers, male opening flowers, and fruits at three different developmental stages. The data showed that CsHAN1 has the highest level in the young fruits 4 d before anthesis, and exhibited the lowest level in the male opening flowers (Fig. 2A). The expression of CsHAN1 in floral buds is higher than that in the opening flowers (Fig. 2A), suggesting that CsHAN1 is more abundant in young tissues.

To investigate the spatial and temporal expression pattern of CsHAN1, in situ hybridization was performed. The signal of CsHAN1 is detected at the junction of the inflorescence meristem (IM) and the stem (arrow in Fig. 2B), the junction regions of the floral meristem (FM) and the stem (asterisks in Fig. 2C–E), and in the axil of leaf primordia (arrowhead in Fig. 2B). In addition, transcripts of CsHAN1 are mainly concentrated in the boundary between petal primordia and stamen primordia, and the boundary between stamen primordia and carpel primordia in the stage 4 flower bud (arrows in Fig. 2E). In the male flower, CsHAN1 is primarily expressed in the developing anthers at stage 9 (Fig. 2F), and then in the tapetum cell layer and uninuclear pollen at stage 11 (Fig. 2G). In the female flower, the expression domain of CsHAN1 is mostly in the developing ovary (Fig. 2H), ovules (Fig. 2I), and the base of the embryo sac (Fig. 2J). CsHAN1 is also strongly expressed in vascular tissues in all of the examined samples (arrows in Fig. 2I, J). No signal is detected upon hybridization with the sense CsHAN1 probe (Fig. 2K).

Ectopic expression of CsHAN1 in Arabidopsis

To investigate the function of CsHAN1, Arabidopsis was first ectopically introduced under the Cauliflower mosaic virus (CaMV) 35S promoter into the han-2 mutant in the Ler background. However, only two transgenic plants were produced, screened from ~3ml (1.1 × 10⁵) seeds of seeds, and died without producing any seeds (Supplementary Fig. S1A, B at JXB online). This is similar to the overexpression of AtHAN itself in Arabidopsis (Zhao et al., 2004). Next, CsHAN1 was ectopically expressed in the han-2 mutants in the Col background which displays similar reduced floral organs and decreased siliques length to those in the Ler background (Zhang et al., 2013). Fortunately, 17 independent transgenic lines were produced, and the degree of rescue of the han-2 mutant phenotype positively correlates with the ectopic CsHAN1 expression (Fig. 3A–J) (investigation of the phenotype was performed in the T₂ transgenic lines). For example, the number of petals rescued ranged from 1.9 ± 1.1 in han-2 to 3.6 ± 0.5 in the strongest transgenic line 5, and 3.1 ± 0.9 in the weakest line 6 (Fig. 3C–E; Table 1). Similarly, the length of the siliques in the three CsHAN1 transgenic lines was also increased, and line 5 recovered almost to the length of the WT (Fig. 3F). In addition, in contrast to the slightly serrated margin in Col, it was noticed that the rosette leaves of han-2/Coll are spindly with smooth margins and short petioles (Fig. 3G, H), and CsHAN1 can rescue the smooth leaves to serrated upon ectopic expression in Arabidopsis (Fig. 3I). These results suggest that CsHAN1 may play a role in regulation of flower organ and leaf shape development.

To explore further the function of CsHAN1, transgenic lines overexpressing CsHAN1 in Arabidopsis WT Col were also generated. A total of 12 independent transgenic lines were obtained. Overexpression of CsHAN1 leads to serrated leaves in both rosette leaves and cauline leaves, and produces short siliques that may partially result from the 28% short stamens that are not long enough to reach the stigma and/or immature anthers (Fig. 3K–N; Supplementary Fig. S1C at JXB online). However, the number of floral organs is unchanged in the CsHAN1 overexpression lines.

CsHAN1 may be involved in shoot apical meristem development in cucumber

To understand further the function of CsHAN1 in cucumber, the 35S promoter followed by the CsHAN1 coding sequence (CsHAN1-OE) or the double-stranded RNAi construct containing the specific sequence of CsHAN1 (CsHAN1-RNAi) was introduced into the cucumber inbred line R1407 through Agrobacterium-mediated cotyledon transformation, and positive transplants were selected based on antibiotic selection as well as PCR analyses using primers from the vector (Y. Zhang et al., 2014; Cheng et al., 2015). Nine CsHAN1-OE and 11 CsHAN1-RNAi independent T₀ transgenic lines were obtained. Surprisingly, the expression of CsHAN1 was down-regulated in the CsHAN1-OE lines whereas it was up-regulated in the CsHAN1-RNAi lines (Fig. 4A; Supplementary Fig. S2 at JXB online), which can be explained by co-suppression in the CsHAN1-OE lines and negative autoregulation of HAN in the CsHAN1-RNAi lines (Baudry et al., 2006; Zhang et al., 2013). Next three representative transgenic lines for each construct were selected for further characterization (Fig. 4A). In the three T₀ CsHAN1-OE lines, transcripts of CsHAN1 declined to 30, 31, and 40% in lines 9, 12, and 15, respectively, as compared with those in the empty vector (WT). In the T₀ CsHAN1-RNAi lines, the expression of CsHAN1 is up-regulated 1.6- to 3-fold (Fig. 4A). Line 9 of CsHAN1-OE grew slowly, with very few flower buds and lobed leaves (Fig. 4B; Supplementary Fig. S3), and it died after 3 months without generating any seeds. Despite CsHAN1-OE lines 12 and 15 producing several seeds, the resulting T₁ plants display severely retarded growth and appear to be sterile (no seeds produced after pollination). Similarly, the T₀ CsHAN1-RNAi line also grew slowly (Fig. 4B; Supplementary Fig. S3). The stunted transgenic lines suggest that CsHAN1 may function in SAM development. To confirm this notion, embryo development was characterized in the T₂ CsHAN1-RNAi lines (Fig. 5). In the WT, the cucumber embryo developed to the torpedo stage and the meristem protruded upward, forming a dome at 16 d after pollination (Fig. 5A) (Atsmon and Galun, 1960; Sun et al., 2010). In the CsHAN1-RNAi line 49, ~30% of embryos remained at the heart stage (Fig. 5B), and 60% embryos were in the torpedo stage with a flat meristem (Fig. 5C). In the CsHAN1-RNAi line 90, although 75% of embryos were at the torpedo stage, the meristem was small or did not fully protrude (Fig. 5D). Next, the rate of seed germination was compared for 36h; the root of WT cucumbers was ~3cm long (Fig. 5E), while the root in the CsHAN1-RNAi lines just began to emerge or was <2cm long (Fig. 5E). The seed morphology was also affected in
the CsHAN1-RNAi lines, with 39% of seeds obviously crapy and smaller than those in the WT (Fig. 5F). Therefore, CsHAN1 can retard plant growth early after embryogenesis.

Given that WUS was shown to be a classical meristem marker that functions in specifying the stem cell identity in the shoot meristem, and STM and BP are KNOX family genes that promote meristem maintenance (Long et al., 1996; Byrne et al., 2002; Douglas et al., 2002; Lenhard et al., 2002), it was next explored whether HAN suppresses SAM development through these genes. qRT–PCR analyses
Table 1. CsHAN1 can partly rescue the number of floral organs in the han-2 mutant in Arabidopsis

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sepal</th>
<th>Petal</th>
<th>Stamen</th>
<th>Carpal</th>
</tr>
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<tbody>
<tr>
<td>Col</td>
<td>4.0±0.0</td>
<td>4.0±0.0</td>
<td>5.5±0.3</td>
<td>2.0±0.0</td>
</tr>
<tr>
<td>han-2(Col)</td>
<td>3.6±0.6</td>
<td>1.9±1.1</td>
<td>4.5±0.6</td>
<td>2.0±0.0</td>
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<tr>
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<td>3.8±0.5</td>
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<td>4.5±0.5</td>
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<tr>
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<td>3.1±0.9</td>
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<td>2.0±0.0</td>
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The values shown are the means ±SE, n=30.

showed that the expression of CsWUS, CsSTM, and CsBP was greatly decreased in the shoot apexes of CsHAN1-RNAi lines (Fig. 5G). In situ hybridization of CsWUS, CsSTM, and CsBP was also performed in the shoot apexes of CsHAN1-RNAi lines. The CsWUS signal was detected in the organizing center of the SAM, IM, and FM in the WT, consistent with findings in other species (Fig. 5H), while the expression of CsWUS was significantly reduced in the CsHAN1-RNAi line (Fig. 5I). CsSTM is expressed throughout the SAM and FM but not in the organ primordia in the WT (Fig. 5J). In the CsHAN1-RNAi line, the CsSTM signal is also greatly decreased (Fig. 5K). Similarly, CsBP mRNA is detected at the base of the SAM and the cortex of the stem in the WT (Fig. 5L), and the transcript level of CsBP is greatly reduced in the CsHAN1-RNAi line (Fig. 5M). These data suggested that CsHAN1 may regulate meristem development by mediating the expression of CsWUS, CsSTM, and CsBP in cucumber.

CsHAN1 regulates leaf shape development in cucumber

In addition to the retarded growth, another obvious phenotype in the CsHAN1 transgenic cucumber was the lobed leaves (Fig. 6). In contrast to the palmate entire leaves in the WT, a high proportion of the leaves in both CsHAN1-OE and CsHAN1-RNAi lines were highly lobed (Fig. 6A–F), especially in leaves at the first 10 nodes, probably due to different penetration and developmental cues at different nodes (Fig. 6G) (Weigel et al., 1992; Ji et al., 2011). To explore the mechanism by which CsHAN1 regulates leaf shape development in cucumber, the known leaf developmental genes in cucumber were first isolated using a BLAST search, and then the expression in the fourth young leaves was examined by qRT–PCR in the T2 plants. The expression of CsJAG, CsBP, and CsKAT6 was down-regulated in the CsHAN1-OE lines and up-regulated in the CsHAN1-RNAi lines, whereas the expression of CsAGO1 and CsKAT2 was reduced in both the CsHAN1-OE lines and CsHAN1-RNAi lines (Fig. 6H). The expression of CsPNH2 was reduced >2-fold in the CsHAN1-RNAi lines, but was unchanged in the CsHAN1-OE lines (Fig. 6H). The expression of CsPNH1 and CsAS2 appears to be unaffected in both transgenic lines (Fig. 6H), suggesting that CsHAN1 regulates leaf shape development through a complicated gene regulatory network in cucumber.

Discussion

CsHAN1 may regulate shoot meristem development through regulating WUS and STM pathways in cucumber

In Arabidopsis, WUS and STM function in independent pathways and play essential roles for SAM establishment and maintenance (Lenhard et al., 2002). Here it was found that both CsHAN1-OE and CsHAN1-RNAi lines displayed retarded growth, but CsHAN1-OE lines displayed a more severe phenotype than the CsHAN1-RNAi lines, probably due to the huge reduction caused by co-suppression in the CsHAN1-RNAi lines (Figs 4–6). In situ hybridization showed that the expression of CsWUS was significantly reduced in the CsHAN1-RNAi lines, despite the fact that the expression domain remained unchanged (Fig. 5H, I). However, the expression of AthWS was diffused and shifted to the L2 or L1 layer in the han-1 mutant plants in Arabidopsis (Zhao et al., 2004), implying that CsHAN1 and AthAN may regulate WUS in a different way. In addition, embryo development in the han-1 mutant was uncoordinated in Arabidopsis,
resulting in misshapen embryos (Zhao et al., 2004; Navy et al., 2010), whereas the embryo development in the CsHAN1-RNAi line was delayed, with no obvious change of embryo shape. There are two possibilities to explain this difference: one is that CsHAN1 and AthAN regulate embryo development through a distinct mechanism, and the other
possibility is that the embryo defects in the CsHAN1-RNAi line were covered by CsHAN1 autoregulation; the 1.6- to 3-fold increase of CsHAN1 expression in the CsHAN1-RNAi line was within the buffer threshold that fails to produce any morphological defects in the embryo. A clean loss of function of CsHAN1 like that in the han-1 null allele would better elucidate the function of CsHAN1 in embryo development in cucumber.

Further, it was found that the expression of CsSTM and CsBP was greatly reduced in the CsHAN1-RNAi lines (Fig. 5J–M). STM and BP both belong to the class 1 KNOX genes, and were shown to function redundantly in meristem maintenance in Arabidopsis (Byrne et al., 2002; Douglas et al., 2002; Lenhard et al., 2002). Given that the expression domain of CsHAN1 overlaps with that of CsWUS, CsSTM, and CsBP (Figs 2, 5), CsHAN1 may regulate meristem development through physical interactions with CsWUS and CsSTM, bridging the previously speculated two parallel pathways in cucumber. Further studies using inducible CsHAN1 lines and ChIP assay will be helpful to test the above hypothesis.

Elaborate expression of CsHAN1 is required for normal leaf shape development

Previous studies of HAN emphasized its role in flower and embryo development (Zhao et al., 2004; Nawy et al., 2010), while the function of HAN in leaf development was largely neglected. Here it was found that leaves of the han-2 mutant in the Col background changed from serrated into smooth margins (Fig. 3G, H), Together with the finding that ectopic expression of AtHAN led to lobed leaves in Arabidopsis (Zhao et al., 2004), a function for AtHAN in leaf shape development is hypothesized.

In this study, it was found that ectopic expression of CshAN1 can rescue the smooth margin phenotype in the han-2 mutant and resulted in lobed leaves in WT Arabidopsis (Fig. 3). More importantly, both CshAN1-OE and CshAN1-RNAi lines produced highly lobed leaves in cucumber (Fig. 6A–F), especially in the leaves at the first 10 nodes (Fig. 6G). The CUC boundary genes have been shown to play a role in leaf development (Aida et al., 1999; Nikovics et al., 2006; Hasson et al., 2011). In tomato, both reduction and overexpression of the
CUC homologous gene GOBLET (GOB) led to a change from complex leaves in the WT into simpler leaves with no sharp leaf margin (Blein et al., 2008; Berger et al., 2009). These data suggest that the elaborate expression of the boundary genes HAN and CUC is essential for leaf shape development, with increased or reduced expression resulting in a change in leaf margins. However, molecular and genetic studies are required to establish whether HAN and CUC may be part of the same pathway or act independently in leaf development.

Further, the present data showed that despite the CsHAN1-OE lines and CsHAN1-RNAi lines displaying similar leaf phenotype, the underlying gene expression was different (Fig. 6). As a co-suppression event may be involved in the CsHAN1-OE lines and negative autoregulation of AtHAN has been well documented (Zhang et al., 2013), the final phenotypes of transgenic plants might derive from different levels of HAN proteins. The expression of CsJAG, CsBP, CsKNAT2, and CsKNAT6 was down-regulated in the CsHAN1-OE lines (Fig. 6). Interestingly, JAG, BP, KNAT2, and KNAT4 were also shown to be down-regulated upon AtHAN induction in Arabidopsis (Zhang et al., 2013), suggesting that a similar regulatory mechanism may be involved between HAN, JAG, BP, and KNOX genes in cucumber and Arabidopsis. CsBP was found to be down-regulated in the meristem but up-regulated in the leaves of CsHAN1-RNAi cucumber plants, implying that different regulatory networks exist in different tissues and/or developmental stages.

**Supplementary data**

Supplementary data are available at JXB online.

**Figure S1.** CsHAN1 overexpression in WT Arabidopsis.

**Figure S2.** PCR identification and qRT-PCR analyses of transgenic cucumber.

**Figure S3.** Leaf phenotype in the CsHAN1 transgenic cucumber.

**Figure S4.** Flower morphology in Arabidopsis and cucumber.

**Table S1.** Primers used in this study.

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