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HEN1 functions pleiotropically in *Arabidopsis* development and acts in C function in the flower

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SUMMARY

Four classes of floral homeotic MADS domain proteins specify the identities of the four organ types in an *Arabidopsis* flower. While the activities of the MADS domain proteins are essentially confined to the flower or to the inflorescence, several genes, such as *APETALA2, HUA1* and *HUA2*, also act outside the flower in addition to their organ identity functions inside the flower. We identified a new gene, *HUA ENHANCER 1* (*HEN1*) from a sensitized genetic screen in the *hua1-1 hua2-1* background that is compromised in floral homeotic C function. We showed that *HEN1*, like the C function gene *AGAMOUS*, acts to specify reproductive organ identities and to repress A function. *HEN1* also shares *AG*'s non-homeotic function in controlling floral determinacy. *HEN1* may achieve these functions by regulating the expression of *AG*. *hen1* single mutants exhibit pleiotropic phenotypes such as reduced organ size, altered rosette leaf shape and increased number of coflorescences, during most stages of development. Therefore, *HEN1*, like the A function gene *AP2*, plays multiple roles in plant development as well as acting in organ identity specification in the flower. *HEN1* codes for a novel protein and is expressed throughout the plant.

Keywords

Flower development; Organ identity; C function; *HUA1*; *HUA2*; *HEN1*; *AGAMOUS*

INTRODUCTION

Three classes of floral homeotic genes, known as the A, B and C genes, act in overlapping domains in the floral meristem to specify the identities of the four floral organ types (Coen and Meyerowitz, 1991; Meyerowitz et al., 1991). While the A, B and C genes each act in two adjacent whorls, the *SEPALLATA* (*SEP*) *1, 2* and *3* genes (also referred to as the class E genes) act in whorls 2, 3 and 4 to specify petal, stamen and carpel identities together with the A, B and C genes (Pelaz et al., 2000; Theißen and Saedler, 2001).

With the exception of the A function gene *APETALA2* (*AP2*), the A, B, C and E genes code for MADS domain DNA-binding proteins (Yanofsky et al., 1990; Ma et al., 1991; Jack et al., 1992; Mandel et al., 1992; Shiraishi et al., 1993; Huang et al., 1993; Goto and Meyerowitz,

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1994; Jofuku et al., 1994; Riechmann et al., 1996; Mandrel and Yanofsky, 1998) that likely act as transcription factors in multimeric complexes (Gutierrez-Cortines and Davies, 2000; Honma and Goto, 2001). The expression of the floral homeotic MADS box genes is largely restricted to the flower and to the specific floral whorls where they function (Yanofsky et al., 1990; Drews et al., 1991; Ma et al., 1991; Jack et al., 1992; Mandrel et al., 1992; Goto and Meyerowitz, 1994; Mandrel and Yanofsky, 1998). In fact, ectopic expression of the floral homeotic MADS box genes in leaves can convert leaves to floral organs (Honma and Goto, 2001; Pelaz et al., 2001), suggesting that the A, B, C and E MADS box genes constitute most, if not all, of the flower-specific regulators of floral organ identities. However, genes that play pleiotropic roles in the development of the plant may also serve as regulators of floral organ identities. One such example is the A function gene *AP2* (Jofuku et al., 1994; Okamuro et al., 1997).

AG is essential for stamen and carpel identities and floral determinacy. *AG* also antagonizes A function by restricting the activities of *AP2* and the expression of *APETALA1* (*API*) to the outer two floral whorls (Bowman et al., 1991b; Gustafson-Brown et al., 1994). *AG* controls floral determinacy by repressing the expression of *WUSCHEL*, a gene that specifies stem cell fates, in the center of the flower (Schoof et al., 2000; Lenhard et al., 2001; Lohmann et al., 2001). How *AG* specifies stamen and carpel identities at the molecular level is less well understood. Since AG likely functions in complexes involving the B and E proteins, the B, C and E MADS domain proteins probably act at the same developmental hierarchy in the floral homeotic pathways.

Several other genes that act in the specification of carpel and/or stamen identities have been identified. *CRABS CLAW* (*CRC*), encoding a protein with zinc finger and helix-loop-helix domains, and *SPATULA* (*SPT*), encoding a basic helix-loop-helix protein, control aspects of gynoecium development (Alvarez and Smyth, 1999; Bowman and Smyth, 1999; Heisler et al., 2001). Either *crc-1* or *spt-2* reduces the carpelloidy present in the first whorl organs of *ap2-2 ag-1* flowers, suggesting that the two genes also function in carpel identity specification (Alvarez and Smyth, 1999). Two other genes, *HUA1* and *HUA2*, were found to control stamen and carpel identities, antagonize A function, and specify floral determinacy (Chen and Meyerowitz, 1999). These functions of *HUA1* and *HUA2* were only recognized in genetic backgrounds in which *AG* activities are compromised, such as in the weak *ag-4* allele (Sieburth et al., 1995) and in the *ag-1/+* background. *HUA2* codes for a novel protein (Chen and Meyerowitz, 1999) that has transcription activation activity (unpublished results), and *HUA1* codes for a nuclear RNA-binding protein with CCCH zinc fingers (Li et al., 2001). The molecular relationship among *HUA1, HUA2* and *AG* has yet to be determined.

In order to identify additional players that specify reproductive organ identities and/or control floral determinacy, we performed a genetic screen in the *hua1-1 hua2-1* double mutant background. Two recessive mutations in the *HUA ENHANCER 1* (*HEN1*) locus strongly enhance the *hua1-1 hua2-1* homeotic phenotypes in the inner two whorls. The *hen1* mutations also convert the determinate *hua1-1 hua2-1* floral meristems to indeterminate meristems, suggesting that *HEN1* also controls floral determinacy. The *hen1* mutant plants have shorter stems and smaller leaves that differ in shape from those of wild type. Therefore, *HEN1* is a gene that plays multiple roles in plant development.

MATERIALS AND METHODS

Plant strains and EMS mutagenesis

The strains used in this study are all in the Landsberg *erecta* (L*er*) background, except that wild-type Columbia plants were used for mapping. *ag-4* (Sieburth et al., 1995), *ap1-1* (Irish and Sussex, 1990; Bowman et al., 1993), *ap2-2* (Bowman et al., 1991b), *hua1-1* (Chen and Meyerowitz, 1999) and *hua2-1* (Chen and Meyerowitz, 1999) have been characterized previously. *hen1-1* and *hen1-2* were isolated in this study.

An EMS mutagenesis screen was carried out in the *hua1-1 hua2-1* background. 10,000 *hua1-1 hua2-1* seeds were treated with 0.2% EMS/0.01% Tween 20 for 12 hours, and then planted onto soil. M_2 seeds were collected from single M_1 plants. Among 1551 M_2 families screened for floral homeotic phenotypes in the inner two whorls, two lines were isolated that were later found to contain mutant alleles at the *HEN1* locus. *hen1-1* was backcrossed to L*er* three times before further genetic analysis while *hen1-2* was backcrossed twice.

Complementation tests and construction of mutant combinations

The *hua1-1 hua2-1 hen1-1* and *hua1-1 hua2-1 hen1-2* plants show complete male sterility and extremely reduced female fertility. For complementation tests, we first crossed each triple mutant to *hua1-1 hua2-1* to obtain *hua1-1 hua2-1 hen1-1/+* and *hua1-1 hua2-1 hen1-2/+* plants. Plants of these two genotypes were then crossed to each other. Among 29 F_1 plants from this cross, 7 showed the triple mutant floral and vegetative phenotypes, suggesting that the two mutations are allelic.

The *hua1-1 hua2-1 hen1-1 ap1-1* mutant was generated by crossing *hua1-1 hua2-1 hen1-1/+* plants to *hua1-1 hua2-1 ap1-1* plants. In the F_2 population, plants that were homozygous for *hen1-1* were first identified by their vegetative phenotype and examined for the presence of *ap1-1*-like floral phenotypes (the presence of flowers at the base of the first whorl organs and lack of second whorl petals) to identify the quadruple mutant. Similarly, *ap2-2* was introduced into the *hua1-1 hua2-1 hen1-1* background. In this case, a total of 336 F_2 plants were screened to obtain three quadruple mutants because *HEN1* and *AP2* are linked by approximately 20 cM.

Map-based cloning of HEN1

hua1-1 hua2-1 hen1-1/+ plants were crossed to wild-type plants of the Columbia ecotype. F_2 seeds were harvested from single F_1 plants. The F_2 families that segregated *hen1-1* were identified by the presence of the *hen1-1* vegetative and the *hua1-1 hua2-1 hen1-1* floral phenotypes. In these F_2 families, 796 F_2 plants that were homozygous for *hen1-1* were identified with the vegetative phenotypes. Genomic DNA was isolated from each of the 796 *hen1-1* plants. Initial mapping with 40 such plants using simple sequence length polymorphism (SSLP) and cleaved amplified polymorphic sequences (CAPS) markers located *HEN1* to chromosome IV. Additional CAPS markers based on polymorphisms between L*er* and Col provided by Cereon allowed the mapping of *HEN1* to a single BAC. Information on these markers can be obtained at the Chen lab web site (http://waksman.rutgers.edu/~xuemei).

HEN1 cDNA and genomic clones

The *HEN1* full-length cDNA sequence from the WS ecotype was already deposited into GenBank under the name *GENEY* in a study that was focused on its neighboring gene, *POLLENLESS3* (Sanders et al., 1999). We screened the Weigel floral cDNA library and obtained a partial *HEN1* cDNA from the L*er* ecotype. The 5′ missing portion of the L*er* cDNA was obtained by reverse transcription reactions followed by polymerase chain reactions (RT-PCR). The sequence of the *HEN1* cDNA from L*er* agrees with the published sequence of *GENEY* except for three nucleotides, which are likely due to ecotype polymorphisms. The *HEN1* cDNA from L*er* is in GenBank under the accession number AF411383.

For complementation tests, the *HEN1* genomic region was amplified from the BAC T13K14 with Ex-Taq (Panvera) using primers HEN1p10 (5′ *Kpn*I-tcatggattcgtggtatagcgttactt 3′) and HEN1p11 (5′ *Kpn*I-gccctcgtgaaaaagatcaagaacgc 3′) and cloned into pPZP211 (Hajdukiewicz et al., 1994) to result in pPZP211-HEN1p10/p11. This clone contains the entire *HEN1* genomic region with 1847 bp upstream of the initiation codon but excludes the neighboring genes on either side. This clone was used to transform *hen1-1* and *hua1-1 hen1-1 hua2-1/+* plants using vacuum infiltration (Bechtold et al., 1993).

Scanning electron microscopy

Tissues were fixed and critical point dried according to the method of Bowman et al. (Bowman et al., 1991b). Images were captured with an AMRAY-1830 I microscope.

RNA filter and in situ hybridization

Total and poly(A)$^+$ RNA isolation was carried out as described previously (Chen and Meyerowitz, 1999). For RNA filter hybridization, approximately 1 μg of poly(A)$^+$ RNA was used for each sample. A 978 bp region of the *HEN1* cDNA was amplified by PCR with T13K14p21 (5′-TCA GGA TCC ACT GCC AAAGA-3′) and T13K14 p22 (5′-GTT TGG CAA AGC TTC CTGTG-3′) and used as the *HEN1* probe.

In situ hybridization with radioactive probes was carried out as described previously (Chen and Meyerowitz, 1999). In situ hybridization with digoxigenin (DIG)-labeled probes was carried out according to the protocol described at www.wisc.edu/genetics/CATG/barton/protocols.html. The plasmids used for *AG, AP1, AP3, PI, SEP1, SEP2* and *SEP3* probes are pCIT565 (Yanofsky et al., 1990), pKY89 (Gustafson-Brown et al., 1994), pD793 (Jack et al., 1992), pcPINX (Goto and Meyerowitz, 1994), pCIT4221 (Ma et al., 1991), pSR12 (Savidge et al., 1995) and pAGL9 (Mandrel and Yanofsky, 1998), respectively. For *HEN1*, the 978 bp T13K14p21/p22 fragment was amplified with Pwo polymerase and cloned into pCR-BluntII-TOPO (Invitrogen) in two orientations to result in pTOPO-cHEN1S and pTOPO-cHEN1A. pTOPO-cHEN1S and pTOPO-cHEN1A were digested with *Bam*HI and *Hin*dIII, respectively, and transcribed with T7 polymerase for generating the sense and antisense probes.

RESULTS

hen1 single mutant phenotypes

The *hua1-1 hua2-1* double mutant exhibits vegetative as well as floral phenotypes: slightly smaller leaves, shorter stems and flowers with weak carpel-to-sepal transformation (Chen and Meyerowitz, 1999; see below). An EMS genetic screen was carried out to search for mutations that enhance the *hua1-1 hua2-1* floral phenotypes. Among 1551 M_2 families screened, two recessive *hen1* alleles, *hen1-1* and *hen1-2*, were isolated. While the *hua1-1 hua2-1 hen1-1* and *hua1-1 hua2-1 hen1-2* flowers showed severe loss-of-C-function phenotypes (see below), the triple mutant plants were also much smaller than the *hua1-1 hua2-1* double mutant (data not shown), suggesting that *HEN1* functions in vegetative development. In fact, while *hen1-1* and *hen1-2* single mutants lack obvious floral homeotic phenotypes, several non-homeotic defects are clearly visible.

hen1-1 and *hen1-2* plants are smaller in stature compared to wild-type plants. Most aerial organs in *hen1* mutants are reduced in size: leaves and floral organs are smaller (Fig. 1A–C, E), and stems are shorter (data not shown). The *hen1* (throughout the paper, both alleles are referred to when allele numbers are not specified) cotyledons, however, are similar in size to those of wild type (data not shown). In addition to reduced size, *hen1* leaves are shaped differently: they are more pointed at the apical end (Fig. 1B,C) and the edges tend to curl upward (Fig. 1B). Flower density also appears to be much higher in *hen1* inflorescences (Fig. 1F,G). However, this could be a secondary effect of reduced internode elongation in *hen1* stems. Scanning electron microscopy (SEM) revealed that cells in mature *hen1-1* leaves and petals are smaller than those in mature L*er* leaves and petals (Fig. 1H–M).

hen1-1 and *hen1-2* single mutants exhibit delayed transition to producing flowers on the inflorescence stems (Table 1). Under our long-day conditions (16 hour light/8 hour darkness), both *hen1-1* and *hen1-2* plants produce more cauline leaves with axillary inflorescences before producing single flowers on the inflorescence stems than L*er* plants, with *hen1-1* being stronger than *hen1-2*. The two mutations, however, appear to affect rosette leaf number in opposite ways: *hen1-1* plants having a slight increase and *hen1-2* plants having a decrease in rosette leaf number (Table 1).

hen1-1 and *hen1-2* single mutants also exhibit reduced fertility. Male and female fertility both appear to be affected. Anthers from early-arising *hen1-1* and *hen1-2* flowers often fail to dehisce. Although late-arising flowers do produce pollen, the amount of pollen produced appears to be greatly reduced. In addition, when the stigma is pollinated with wild-type pollen, the siliques elongate to the same extent as they would after self-pollination: mature *hen1* siliques reach approximately 1/4–1/2 of the length of a wild-type silique (data not shown). Seed set from *hen1* siliques is accordingly reduced (data not shown).

hen1 mutations enhance the hua1-1 hua2-1 homeotic phenotypes

While *hua1-1 hua2-1* flowers have stamens in the third whorl (Fig. 2A), all flowers of *hua1-1 hua2-1 hen1-1* plants have primarily petals in the third whorl (Fig. 2B,F). Some of the medial third whorl organs occasionally exhibit stamen characteristics, i.e., structures that resemble anther thecae are present (data not shown). *hua1-1 hua2-1 hen1*-2 flowers have

petals and frequently staminoid petals (organs with white pigmentation but containing anther thecae-like structures) in the third whorl (Fig. 2C), suggesting that *hen1-2* is weaker than *hen1-1*. In most *hua1-1 hua2-1 hen1-1* and *hua1-1 hua2-1 hen1-2* flowers, the fourth whorl gynoecia resemble those in *hua1-1 hua2-1* flowers except that the stigmatic papillae usually consist of two or more lobes (Fig. 2B,C). In late-arising flowers, though, the fourth whorl gynoecia can have greatly enlarged ovaries on top of gynophores (Fig. 2D). Inside these ovaries, additional floral organs are present (Fig. 2E). Occasionally, late-arising *hua1-1 hua2-1 hen1-1* flowers resemble those of *ag-3* in that the identities of the two reproductive whorls are completely lost and internal flowers are found in the center (Fig. 2F).

The homeotic transformation in the third whorl and the defect in floral determinacy in late flowers are manifested only in *hua1-1 hua2-1 hen1* triple mutant plants. Like *hua1-1 hua2-1* flowers, *hua1-1 hen1* and *hua2-1 hen1* flowers have stamens and carpels in the inner two whorls (Fig. 2G,H, and data not shown). The *hen1-1* or *hen1-2* single mutant flowers also show normal identities in the four floral whorls (data not shown).

Homeotic transformation at the cellular level

The homeotic transformation in the third whorl of *hua1-1 hua2-1 hen1-1* and *hua1-1 hua2-1 hen1-2* flowers was further confirmed by SEM. Cells that are shaped like pieces of a jigsaw puzzle are found on the surface of wild-type (Smyth et al., 1990) and *hua1-1 hua2-1* third whorl stamens (Fig. 3A). The adaxial and abaxial surfaces of wild-type petals consist of small cone-shaped and cobble stone-like cells, respectively. The epidermal cells of *hua1-1 hua2-1 hen1-1* and *hua1-1 hua2-1 hen1-2* third whorl organs resemble petal cells (Fig. 3B,C). Furthermore, a study of ontogeny revealed that the homeotic transformation in the third whorl of *hua1-1 hua2-1 hen1-1* flowers can be observed as early as stages 7–8, when the stamens assume the first signs of differentiation (Smyth et al., 1990). In stages 7–8 flowers of the *hua1-1 hua2-1* genotype, the third whorl organs become spade-shaped, showing signs of differentiation into the anther and the filament (Fig. 3K). In *hua1-1 hua2-1 hen1-1* flowers of similar stages, however, the third whorl organs are flat, resembling perianth organs in shape (Fig. 3L). In wild-type flowers, the third whorl stamen primordia are initiated earlier than the second whorl petal primordia (Smyth et al., 1990). The third whorl petals develops sooner than the second whorl petals in *hua1-1 hua2-1 hen1-1* flowers (data not shown), suggesting that the third whorl petals still behave as third whorl organs in timing of organ initiation, despite the alteration in identity.

Homeotic transformation in the fourth whorl of *hua1-1 hua2-1 hen1-1* and *hua1-1 hua2-1 hen1-2* flowers was also observed with SEM. The gynoecium can be subdivided into the stigma, the style and the ovary along the longitudinal axis. Epidermal cells characteristic of the stigma and style are found at the expected positions along the gynoecia of *hua1-1 hua2-1 hen1-1* and *hua1-1 hua2-1 hen1-2* flowers (data not shown). Ovary epidermal cells in the two triple mutants, however, differ from those in wild-type and *hua1-1 hua2-1* flowers. Epidermal cells along the wild-type ovary lack epicuticular striations (Fig. 3D and data not shown). While cells on the bottom half of the ovary or those along the medial edges of the top half of the ovary in the *hua1-1 hua2-1* gynoecium are normal (Fig. 3F), cells in the lateral portions of the top half of the ovary (coincident with the bulge in the *hua1-1 hua2-1*

gynoecium) show epicuticular thickenings that resemble sepal cells (Fig. 3E). Large cells that are present in abaxial sepals are also found (Fig. 3E), suggesting a carpel-to-sepal transformation in the upper lateral positions in the *hua1-1 hua2-1* ovary. This partial carpel-to-sepal transformation in *hua1-1 hua2-1* ovaries is enhanced by *hen1-1* and *hen1-2*. In *hua1-1 hua2-1 hen1-1* and *hua1-1 hua2-1 hen1-2* gynoecia, epidermal cells all over the ovaries exhibit epicuticular thickenings (Fig. 3G,H). The ovary cell types appear normal in *hua1-1 hen1*, *hua2-1 hen1* and *hen1* gynoecia (Fig. 3I,J, and data not shown).

Expression of floral homeotic genes in hua1-1 hua2-1 hen1-1 mutants

To begin to understand the molecular basis of the homeotic phenotypes in the triple mutants, we studied the expression of the A, B, C and E genes in the mutants. While the expression of classe B (*APETALA3* and *PISTILLATA*) and class E genes (*SEP1*, *2*, *3*) was not detectably different in *hua1-1 hua2-1 hen1*, *hua1-1 hua2-1* and wild-type flowers (data not shown) (Jack et al., 1992; Flanagan and Ma, 1994; Goto and Meyerowitz, 1994; Savidge et al., 1995; Mandrel and Yanofsky, 1998), significant difference was detected for classes A and C gene expression between *hua1-1 hua2-1 hen1* and *hua1-1 hua2-1*.

Class A: AP1

AP1 is expressed throughout stages 1–2 flower primordia and becomes restricted to the outer two whorls starting at stage 3, when *AG* is expressed in the inner two whorls and acts to repress *AP1* expression (Mandrel et al., 1992; Gustafson-Brown et al., 1994). In *hua1-1 hua2-1* flowers, *AP1* expression patterns are similar to those in wild type in stages 1-6 flowers (Chen and Meyerowitz, 1999) (Fig. 4A,B). After stage 6, low levels of *AP1* RNA were detected in a patchy pattern in stamens and carpels (Fig. 4C,D) (Chen and Meyerowitz, 1999). The levels of ectopic *AP1* expression were consistently lower than those found in the outer two whorls of *hua1-1 hua2-1* flowers (Fig. 4C and data not shown). In *hua1-1 hua2-1 hen1-1* flowers, ectopic *AP1* expression in the inner two whorls appeared more precocious and extensive. In some stage 3 flowers, the center of the floral meristem expressed low levels of *AP1* RNA (Fig. 4E). Most stages 6–7 flowers contained *AP1* RNA in stamens and carpels (Fig. 4F). By stages 9–10, *AP1* RNA was detected in the fourth whorl gynoecia at levels comparable to those in the outer two whorls (Fig. 4G). In mature flowers, all cells in the ovary walls expressed *AP1* RNA, whereas only some cells in *hua1-1 hua2-1* ovaries expressed *AP1* (Fig. 4H,D).

Class C: AG

AG starts to be expressed in the incipient stamen and carpel primordia at stage 3 and continues to be expressed in the two organ types throughout flower development (Bowman et al., 1991a; Drews et al., 1991). This expression pattern is preserved in *hua1-1 hua2-1* flowers (Chen and Meyerowitz, 1999) (Fig. 4I,K). In *hua1-1 hua2-1 hen1-1* flowers, the initiation and the domain of *AG* expression were unaltered in stages 1–6 flowers (Fig. 4J). However, *AG* signals were barely detectable and often absent from some third and fourth whorl organs in stage 7 and older flowers (Fig. 4L and data not shown).

Genetic interactions of hen1-1 with ap1 and ap2 mutations

To investigate if the homeotic transformation in the third whorl *of hua1-1 hua2-1 hen1-1* flowers is due to ectopic activities of A function genes, we introduced severe loss-of-function mutations in the A function genes into the *hua1-1 hua2-1 hen1-1* background.

hua1-1 hua2-1 hen1-1 ap1-1

In *ap1-1* flowers, second whorl petals are missing and first whorl sepals are transformed to leaves with additional flowers in their axils (Bowman et al., 1993; Irish and Sussex, 1990). The *ap1-1* mutation is largely additive with the *hua1-1* and *hua2-1* mutations: the *hua1-1 hua2-1 ap1-1* flowers resemble *ap1-1* flowers in the outer two whorls and *hua1-1 hua2-1* flowers in the fourth whorl, although the bulge in the gynoecium is somewhat reduced (Chen and Meyerowitz, 1999). When introduced into the *hua1-1 hua2-1 hen1-1* triple mutant, *ap1-1* partially suppresses the stamen-to-petal transformation in the third whorl (compare Fig. 5A and B with C and D, respectively). The third whorl organs in the quadruple mutant resemble stamens in pigmentation and in overall shape, except that the anthers are flattened and enlarged compared to wild-type anthers (Fig. 5D).

hua1-1 hua2-1 hen1-1 ap2-2

In *ap2-2* flowers, the first whorl organs are leaves or are absent in the lateral positions and in the medial positions are carpelloid with stigmatic papillae all along the edges (Bowman et al., 1991b). The carpelloidy of the first whorl organs is largely due to ectopic *AG* activities (Bowman et al., 1991b; Drews et al., 1991). Additional phenotypes of *ap2-2* include suppression of petal development, reduction in stamen number and often unfused carpels (Bowman et al., 1991b). *ap2-2* is largely additive with *hua1-1* and *hua2-1* in the inner three whorls, such that *hua1-1 hua2-1 ap2-2* flowers exhibit reduced organ numbers in the second and third whorls like *ap2-2* and have bulged gynoecia like *hua1-1 hua2-1* (Chen and Meyerowitz, 1999). The first whorl organs in *hua1-1 hua2-1 ap2-2* flowers have greatly reduced carpelloidy: stigmatic papillae are much reduced in number, suggesting that the *HUA* genes play a role in carpel identity specification (Chen and Meyerowitz, 1999).

Flowers of the *hua1-1 hua2-1 hen1-1 ap2-2* genotype resemble *ap2-2* flowers in overall morphology (Fig. 5E). Petals are missing, stamen number is reduced (Fig. 5E), and carpels are often unfused (data not shown). The first whorl organs resembled those in *hua1-1 hua2-1 ap2-2*, in that the number of stigmatic papillae is greatly reduced compared to that in *ap2-2* (Fig. 5E and data not shown). The most noticeable phenotype of the quadruple mutant is that the third whorl organs resemble stamens (Fig. 5F) instead of petals, which are found in *hua1-1 hua2-1 hen1-1* flowers (Fig. 5A,B). However, the third whorl stamens in the quadruple mutant are not completely wild type: the anthers often have reduced number of thecae and are green on the abaxial side (Fig. 5F and data not shown).

Cloning of HEN1

A map-based strategy was used to clone *HEN1*. *HEN1* was first mapped to a few centimorgans south of *AG* on chromosome IV (Fig. 6A). Detailed mapping with CAPS markers developed from the Cereon SNPs localized *HEN1* to a single BAC, T13K14. Three genes from this BAC were sequenced from *hen1-1*. A C-to-T mutation was identified in one

of the genes, T13K14.80. This mutation would cause a premature stop codon in the predicted protein sequence (Fig. 6B). This gene was then sequenced from *hen1-2*, and a G-to-A mutation was found, which would cause a D-to-N amino acid substitution in the open reading frame (Fig. 6B).

A genomic fragment, HEN1p10/p11, which contained the entire T13K14.80 gene but excluded neighboring genes, was introduced into *hen1-1*. In eight of the nine T_1 transgenic lines, the *hen1-1* defects in leaf size, leaf shape, length of the stem, floral organ size, and fertility were fully rescued (Fig. 1D; data not shown). The increased number of cauline leaves in *hen1-1* was largely rescued, but the increase in rosette leaf number was not (Table 1). Analyses of T_2 populations showed that the T-DNA was responsible for the rescue (data not shown). The same genomic clone was also used to transform *hua1-1 hen1-1 hua2-1*/+ plants (the *hua1-1* and *hua2-1* genotypes were determined with molecular markers). Among 7 T_1 transformants, only one was homozygous for *hua2-1*. This plant exhibited floral phenotypes similar to those in *hua1-1 hua2-1* (Fig. 2I), suggesting that the clone also rescued the floral homeotic phenotype of the *hua1-1 hua2-1 hen1-1* triple mutant. Therefore, T13K14.80 is *HEN1*.

A cDNA corresponding to T13K14.80 from the WS ecotype was published in a study on the function of its neighboring gene, *POLLENLESS3* (Sanders et al., 1999). We obtained and sequenced the full-length *HEN1* cDNA from L*er*. The predicted HEN1 protein sequences from L*er* and WS are nearly identical, with only conservative changes at two amino acids (Fig. 6B). The annotated T13K14.80 sequence from Col differs significantly from the L*er* and WS sequences in a small region (between amino acids 87 and 117). Sequencing of this region in a Col *HEN1* cDNA confirmed that the Col sequence is identical to those from L*er* and WS. Therefore, the difference was the result of mistakes in annotation.

The predicted HEN1 protein does not contain any motifs of known function except for a putative nuclear localization signal (Fig. 6B). One putative protein in the *Arabidopsis* genome shows approximately 50% amino acid identity to HEN1. In fact, this putative gene resides right next to *HEN1*: the initiation codon of *HEN1* and the putative stop codon of this gene are approximately 2.1 kb apart. A transcript from this gene was detected in Col inflorescences by RT-PCR (J. L. and X. C., unpublished result). BLAST searches with the HEN1 protein sequence revealed a region in the HEN1 protein (underlined in Fig. 6B) that shows similarities to predicted proteins from *Caenorhabditis elegans, Drosophila melanogaster, Schizosaccharomyces pombe* and *Streptomyces coelicolor*. Both *hen1* mutations affect this region of the HEN1 protein.

Expression of HEN1

RNA filter hybridization with *HEN1* as the probe detected a single RNA species of the expected size in roots, leaves, stems and inflorescences (Fig. 7). The abundance of *HEN1* RNA is comparable among the different organs (Fig. 7).

In situ hybridization with radioactively labeled *HEN1* probes showed that *HEN1* RNA is present in all cells of the inflorescence meristem, the inflorescence stem, and flowers of all stages (data not shown).

DISCUSSION

HEN1 plays multiple roles in plant development

In addition to causing homeotic transformation in the *hua1-1 hua2-1* background, the two recessive mutations in *HEN1* result in similar and pleiotropic phenotypes during most stages of *Arabidopsis* development in the *HUA1 HUA2* background. The earliest detectable difference between wild-type and *hen1* plants occurs at the end of the 2-leaf stage, when the first pair of *hen1* true leaves do not reach the size of their wild-type counterparts. From this point on, *HEN1* appears to be required for normal growth and development. *HEN1* performs three major functions as revealed by the *hen1-1* and *hen1-2* single mutant phenotypes.

First, *HEN1* is required for most organs, such as leaves, stems, and all four floral organs to achieve their normal size. In leaves and petals, the smaller size in *hen1-1* and *hen1-2* appears to be at least partially due to smaller cells. Therefore, *HEN1* promotes cell expansion. The size of cotyledons appears unaffected by *hen1* mutations.

Second, *HEN1* is required for male and female fertility. This is evident because pollen production in *hen1* mutants is greatly reduced and they exhibit reduced silique elongation and seed set after pollination with wild-type pollen. It remains to be determined what processes in gametophyte development and reproduction are affected by *hen1* mutations.

Third, *HEN1* promotes the transition to flower production on the inflorescence stem. Similar to *leafy* mutants (Weigel et al., 1992), *hen1* mutants make more secondary inflorescences subtended by cauline leaves before producing single flowers on the inflorescence stem. Therefore, like *LEAFY, HEN1* instructs lateral meristems produced from the inflorescence meristem of their floral fate. *HEN1* plays a minor role in floral meristem identity compared to *LEAFY*, since the *hen1-1* phenotype is much weaker than that of *leafy* null mutants. However, *hen1-1* may not be a null allele and therefore the *hen1-1* phenotypes may not fully reflect the function of *HEN1*.

While most aspects of the vegetative and floral phenotypes caused by *hen1-1* and *hen1-2* are consistent, with *hen1-1* being consistently slightly stronger than *hen1-2*, the two mutants differ in the number of rosette leaves. *hen1-2* plants have fewer rosette leaves while *hen1-1* plants have more rosette leaves than wild type. The *hen1-1* rosette leaf number phenotype, however, was not rescued by the *HEN1* genomic clone, suggesting that this phenotype may be due to another mutation still present despite the three backcrosses. If the *hen1-2* rosette leaf number defect is indeed the real *hen1* loss-of-function phenotype, *HEN1* plays a role in delaying the transition from vegetative phase 1 (rosette leaf producing stage) to vegetative phase 2 (cauline leaf producing stage). This conclusion can be verified by further backcrosses of *hen1-1* to wild type and by transforming *hen1-2* with the *HEN1* genomic clone to determine if the rosette leaf number phenotype can be rescued.

HEN1 promotes C function and floral determinacy in flower development

HEN1's role in promoting stamen and carpel identities was revealed only in the *hua1-1 hua2-1* background, in which C function is already affected to some extent. The stamen-to-petal transformation in the third whorl of *hua1-1 hua2-1 hen* flowers is largely due to ectopic

AP2 and *AP1* activities, suggesting that *HEN1* antagonizes A function genes. *HEN1* plays a positive role in organ identity specification in addition to its role in repressing A function because the *hua1-1 hua2-1 hen1-1 ap2-2* and *hua1-1 hua2-1 hen1-1 ap1-1* third whorl organs are not completely stamens. Therefore, *HEN1* promotes C function in the flower, which specifies reproductive organ identities and antagonizes A function.

HEN1 plays a minor role in conferring floral determinacy: late-arising *hua1-1 hua2-1 hen1* flowers can be indeterminate. A number of genes, such as *AG*, *HUA1*, *HUA2* and *CRC*, have been implicated in controlling floral determinacy. *AG* is essential for floral determinacy, as indicated by the indeterminate nature of *ag-2* flowers (*ag-2* is a potential null allele) (Bowman et al., 1991b). That *HUA1* and *HUA2* also play a role in conferring floral determinacy was revealed by the indeterminate nature of *ag-1/+ hua1-1 hua2-1* but not *ag-1/+* flowers (Chen and Meyerowitz, 1999). Similarly, *ag-1/+ crc-1* flowers also exhibit defects in floral determinacy (Alvarez and Smyth, 1999). The fact that *ag-1 hua1-1 hua2-1* flowers are nearly identical to *ag-1* flowers suggests that *ag-1* is epistatic to the *hua* mutations (Chen and Meyerowitz, 1999). In contrast, mutations in *CLAVATA1* and *SUPERMAN* greatly enhance the floral determinacy defect of *ag* mutants (Clark et al., 1993; Schultz et al., 1991). These data suggest that *HUA1* and *HUA2* act in the *AG* pathway to influence floral determinacy. It is unclear whether *CRC* act in the *AG* pathway or in a parallel pathway regarding floral determinacy. Owing to the close linkage between *HEN1* and *AG*, we have not obtained an *ag-1 hen1-1* double mutant to assess if *hen1-1* enhances the floral determinacy phenotype of *ag-1*. An *ag-4 hen1-1* double mutant, however, has been constructed. *hen1-1* does not appear to enhance the floral determinacy defect *of ag-4* (X. C. and J. L., unpublished results). Since *ag-4* and *ag-1* flowers are similar with regard to floral determinacy, it is likely that *HEN1* acts in the *AG* pathway.

Although *HEN1* is involved in the three activities known to require *AG* (specification of reproductive organ identity, repression of A function and control of floral determinacy), *hen1* single mutants do not exhibit any phenotypes that reflect these roles. Severe loss-of-function mutations in *AG*, however, cause complete loss of reproductive organ identity and floral determinacy. If *hen1-1* is a null allele, we can conclude that *HEN1* plays a minor role in reproductive organ identity and floral determinacy specification or that other genes with similar functions can compensate for the loss of *HEN1* function. In fact, in the *HEN1* genomic region, there exists another potential gene that is 50% identical to HEN1 at the protein level. This potential gene may be partially redundant with *HEN1*. However, it is likely that *hen1-1* is not a null allele and therefore its phenotypes do not fully reflect the functions of *HEN1*. A complete loss-of-function mutation in *HEN1* is necessary to assess the full spectrum and extent of *HEN1*'s functions.

HEN1 and AG

The observation that *AG* RNA is reduced in stage 7 and older flowers of the *hua1-1 hua2-1 hen1-1* but not the *hua1-1 hua2-1* genotype suggests that *HEN1* acts to maintain *AG* expression during later stages of flower development. This may be the molecular basis for *HEN1*'s functions in organ identity. In fact, little is known about how *AG* expression is maintained after its initial activation by *LEAFY* and *WUS* (Busch et al., 1999; Lenhard et

al., 2001; Lohmann et al., 2001). *WUS* RNA disappears by stage 6 (Schoof et al., 2000), while *LFY* expression is greatly reduced in the center of the flower after stage 3 (Weigel et al., 1992). *HEN1* is apparently involved in maintaining *AG* expression after stage 6. Since reduction in *AG* expression is not detected in *hen1* single mutants (data not shown), *HEN1* may not be the only gene acting to maintain *AG* expression.

HEN1 may also function as a floral meristem identity gene because *hen1* mutants exhibit delayed transition to making single flowers on the inflorescence stem. We hypothesize that *HEN1* plays a minor role in the initial activation of *AG* early in flower development as well as acting in the maintenance of *AG* expression after the initial activation. The requirement for *HEN1* to activate *AG* expression in early flowers may somehow increase during plant development, such that floral determinacy is lost in late-arising *hua1-1 hua2-1 hen1-1* flowers. This early role of *HEN1* was suggested but not unequivocally demonstrated by in situ hybridization experiments in early flowers (Fig. 4I,J). In summary, we propose that the effects of *hen1* mutations on reproductive organ identity, repression of A function, and floral determinacy are secondary to their effects on *AG* expression. However, it is also possible that the loss of *AG* expression in stages 7 and older flowers in *hua1-1 hua2-1 hen1* flowers is secondary to changes in organ identities.

The fact that most *hua1-1 hua2-1 hen1* flowers exhibit only organ identity but not determinacy defects is apparently contradictory to the previous hypothesis that floral determinacy is more sensitive to the reduction in *AG* expression than organ identity (Mizukami and Ma, 1995; Sieburth et al., 1995). However, this may be explained by the relative timing of reduction of *AG* expression in *hua1-1 hua2-1 hen1-1* and the specification of organ identity and floral determinacy. Owing to the negative regulation by *AG, WUS* RNA disappears from the center of the floral meristem by stage 6 in wild-type plants (Lenhard et al., 2001; Lohmann et al., 2001). Therefore, it is possible that floral determinacy is specified by stage 6, at which point reduction of *AG* expression in *hua1-1 hua2-1 hen1-1* has not commenced or is just about to begin. Conversely, identity specification of the reproductive organs may occur later. Little is known about when *AG* is needed for stamen and carpel identities, but experiments with the temperature sensitive allele *ap3-1* demonstrated that *AP3* function is needed till beyond stage 6 to specify stamen identity (Bowman et al., 1989). Similar temperature shift experiments with the *Antirrhinum def-101* mutant showed that *DEF*, an *AP3* homologue, is required throughout development to maintain petal and stamen identities (Zachgo et al., 1995).

The HEN1 protein

The HEN1 protein sequence does not indicate any molecular function. The fact that the C-terminal region in HEN1 shows similarity to predicted proteins from other organisms and that the two *hen1* mutations both lie in this region suggests that this domain is important for the function of HEN1. The apparently ubiquitous pattern of *HEN1* expression and the pleiotropic phenotypes associated with *hen1* mutations suggest that *HEN1* likely performs a basic molecular function throughout the plant. The specific loss-of-C-function defects in the flower conferred by *hen1* mutations in the *hua1-1 hua2-1* background indicate that either

HEN1 performs a specific function in the flower or that the floral homeotic C function pathway is more sensitive to alterations in the molecular process involving *HEN1*.

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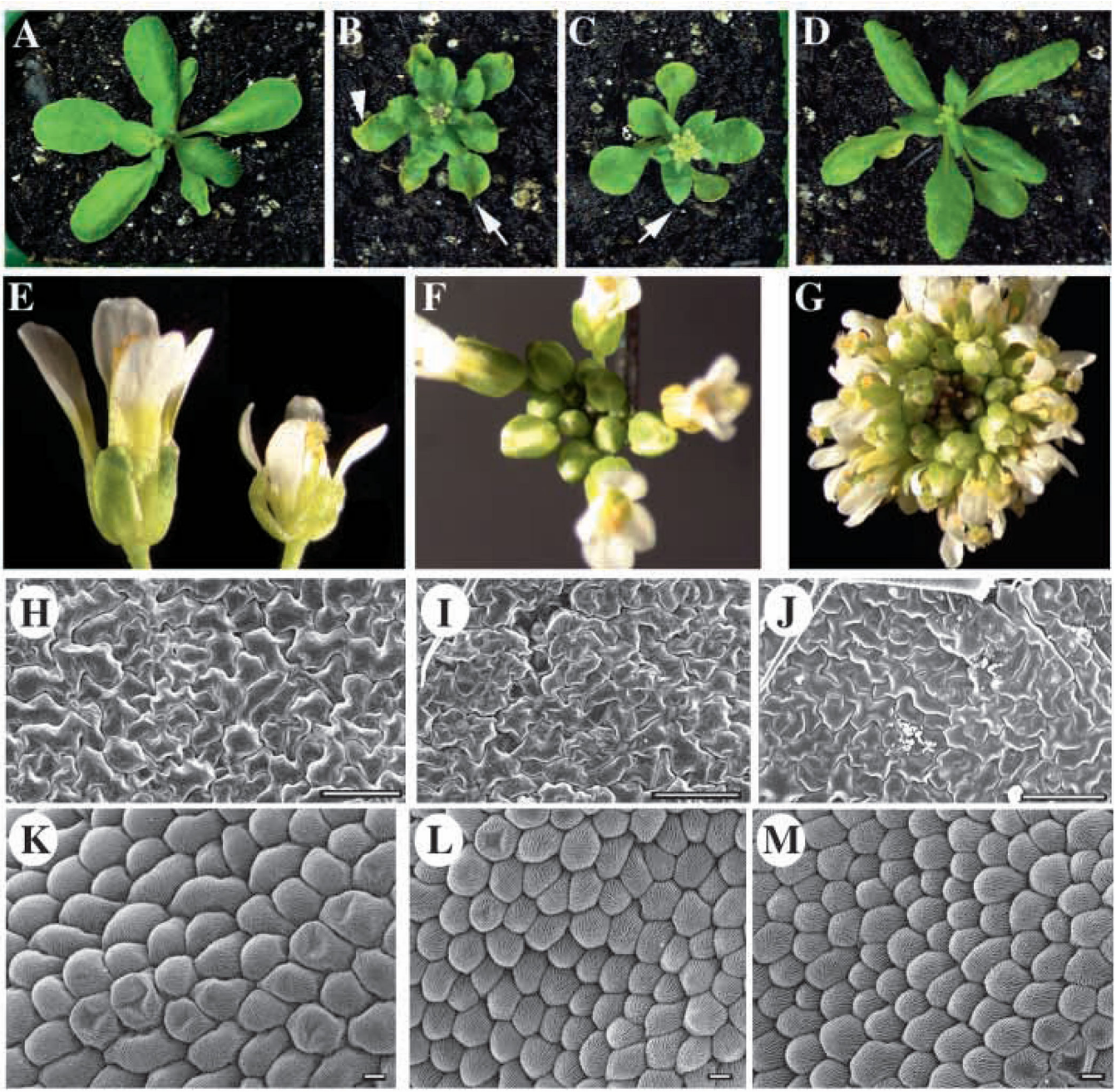

Fig. 1.

hen1-1 and *hen1-2* single mutant phenotypes. (A–C) Rosettes of (A) a L*er*, (B) a *hen1-1* and (C) a *hen1-2* plant, around the time of bolting. Rosette leaves in *hen1-1* and *hen1-2* tend to be pointed (arrows), and the edges tend to curl up slightly (arrowhead). (D) A *hen1-1* plant containing the HEN1p10/p11 transgene. (E) A L*er* (left) and a *hen1-1* (right) mature flower at the same magnification. (F) A L*er* and (G) a *hen1-1* inflorescence. (H–J) Scanning electron micrographs of (H) a L*er*, (I) a *hen1-1* and (J) a *hen1-2* mature leaf. (K–M) Scanning electron micrographs of (K) a L*er*, (L) a *hen1-1* and (M) a *hen1-2* mature petal. Scale bars, 100 μm in H–J, and 10 μm in K–M.

Fig. 2.

Floral phenotypes. (A) A *hua1-1 hua2-1*, (B) a *hua1-1 hua2-1 hen1-1*, and (C) *a hua1-1 hua2-1 hen1-2* flower. (D) A late-arising *hua1-1 hua2-1 hen1-2* flower. The gynoecium is enlarged with a gynophore at the base (arrowhead). (E) A late-arising *hua1-1 hua2-1 hen1-2* flower with additional floral organs (arrowhead) inside the carpels. (F) A late-arising *hua1-1 hua2-1 hen1-1* flower with flowers (arrowhead) in place of carpels. (G) A *hua1-1 hen1-1* and (H) a *hua2-1 hen1-1* flower. (I) A flower of a *hua1-1 hua2-1 hen1-1* plant containing the HEN1p10/p11 transgene. Arrows, third whorl organs. Scale bars, 1 mm.

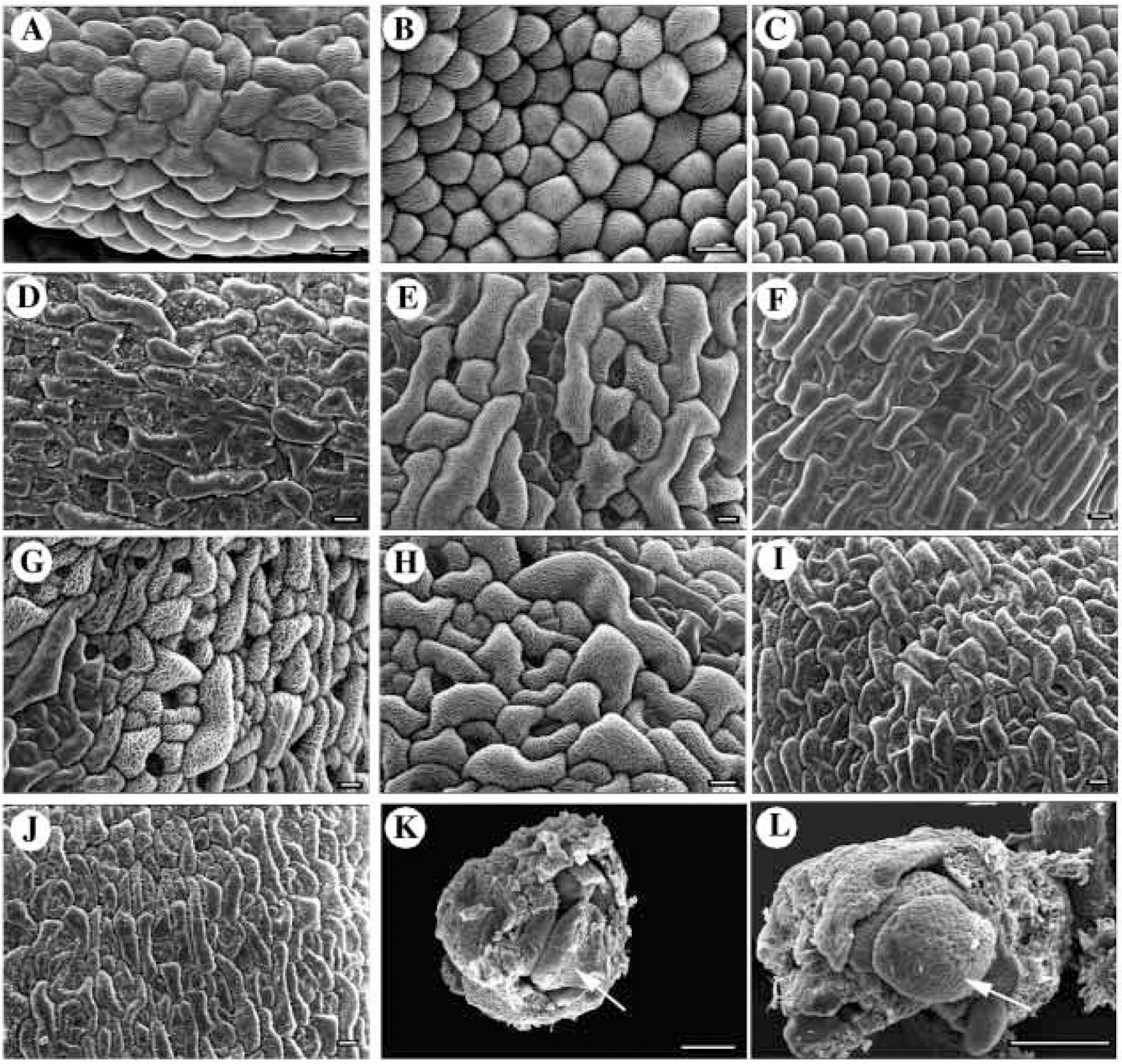

Fig. 3.

Scanning electron microscope images of L*er*, *hen1-1*, *hua1-1 hua2-1*, *hua1-1 hua2-1 hen1-1* and *hua1-1 hua2-1 hen1-2* flowers and/or floral organs. (A–C) The adaxial surface of the third whorl organs from (A) *hua1-1 hua2-1*, (B) *hua1-1 hua2-1 hen1-1* and (C) *hua1-1 hua2-1 hen1-2* flowers. Petal-type cells are found in the third whorl organs in the two triple mutants (B,C). (D) The top portion of a L*er* ovary, showing valve cells that lack cuticular thickenings. (E,F) The tip (E) and the bottom (F) of a *hua1-1 hua2-1* ovary. Some valve cells at the tip of the ovary exhibit cuticular striations that mimic sepal cells (E). The bottom of the *hua1-1 hua2-1* ovary is similar to that in L*er* in terms of cell surface characteristics. (G,H) The bottom portion of ovaries from (G) a *hua1-1 hua2-1 hen1-1* and (H) a *hua1-1*

hua2-1 hen1-2 flower. The cells resemble sepal cells. (I,J) Top portions of the ovaries from (I) a *hua1-1 hen1-1* and (J) a *hua2-1 hen1-1* flower. The cells appear wild type. (K,L) Stage 7–8 flowers of (K) *hua1-1 hua2-1* and (L) *hua1-1 hua2-1 hen1-1* genotypes, with sepals dissected to reveal the third whorl organs (arrows) that differ in shapes. Scale bars, 10 μm except in K and L (100 μm).

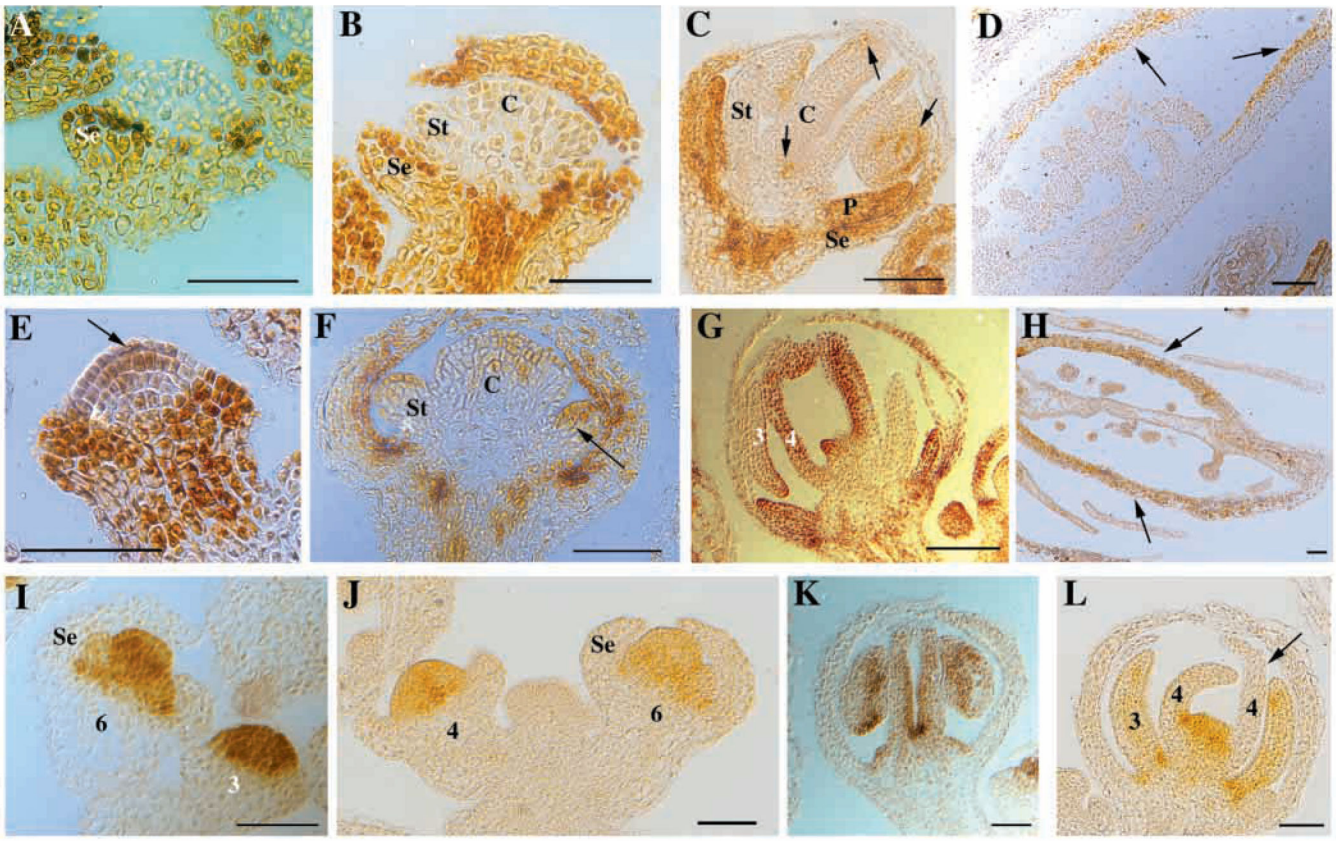

Fig. 4.

AP1 and *AG* RNA accumulation patterns in *hua1-1 hua2-1* and *hua1-1 hua2-1 hen1-1* flowers. (A–H) *AP1* RNA localization in *hua1-1 hua2-1* (A–D) and *hua1-1 hua2-1 hen1-1* (E–H) flowers of various stages. (A,E) Stage 3 flowers; (B,F) stage 6–7 flowers; (C,G) stages 9–10 flowers. (D,H) Mature flowers. Arrows indicate ectopic *AP1* expression. In *hua1-1 hua2-1 hen1-1* flowers, the ectopic *AP1* expression is more precocious (arrows in E and F) and extensive (G and H). (I–L) *AG* RNA localization in (I,K) *hua1-1 hua2-1* and (J,L) *hua1-1 hua2-1 hen1-1* flowers. (I,J) *AG* expression in young flowers (stages indicated by numbers). (K,L) Stage 9 flowers. The arrow in L indicates a fourth whorl organ with little *AG* RNA. Se, sepals; St, stamens; C, carpels. Numbers in G and L indicate floral whorls. Scale bars, 50 μm.

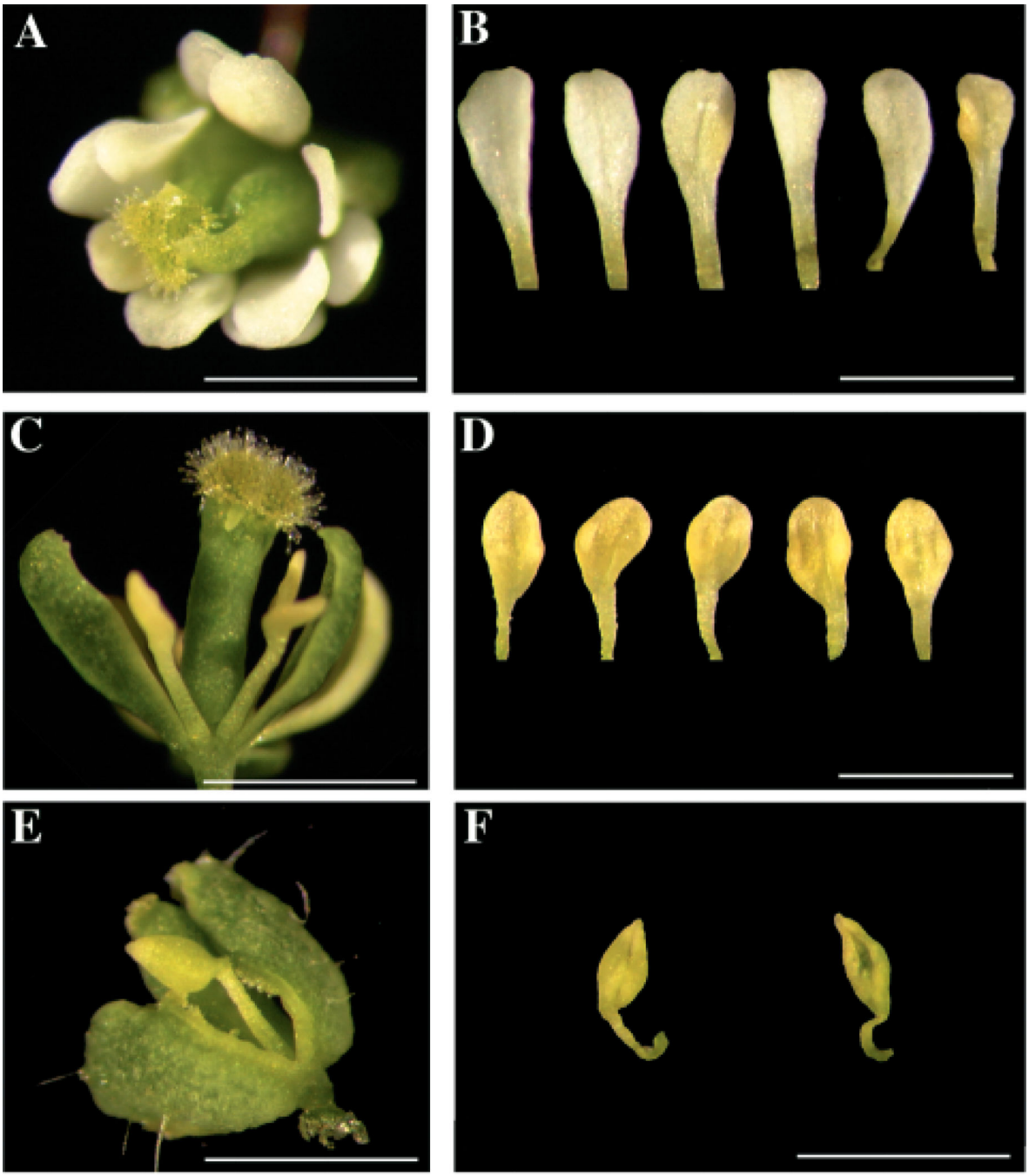

Fig. 5.

Third whorl identity and A function genes. (A) a *hua1-1 hua2-1 hen1-1* flower, (C) a *hua1-1 hua2-1 hen1-1 ap1-1* flower and (E) a *hua1-1 hua2-1 hen1-1 ap2-2* flower. (B,D,F) Dissected third whorl organs from the flowers in A, C and E, respectively. Scale bars: 1 mm.

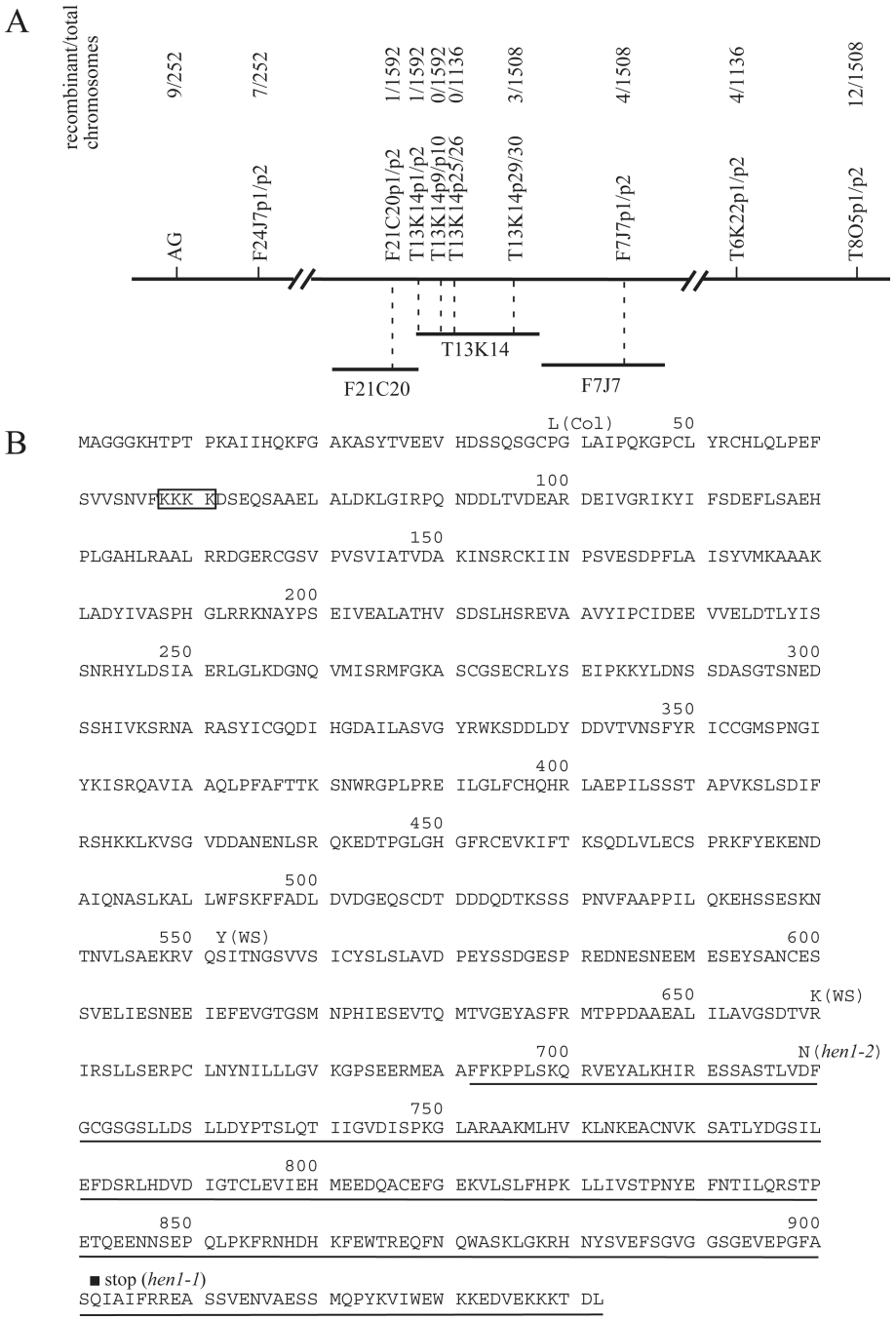

Fig. 6.

Cloning of *HEN1*. (A) Mapping of *HEN1* to the BAC T13K14 on chromosome IV. The *HEN1* genomic region is represented by the long horizontal line. The markers used for mapping are shown above the line with the numbers of recombination break points between *HEN1* and those markers shown. (B) The HEN1 protein sequence from L*er*. *HEN1* codes for a novel protein with a C-terminal domain (underlined) showing similarity to yeast, *C. elegans* and *Drosophila* proteins. The *hen1-1* and *hen1-2* mutations cause truncation and amino acid substitution, respectively, in the C-terminal domain. A potential nuclear

localization signal (NLS) is boxed. The amino acids from Col and WS that differ from those in L*er* are indicated above the sequence.

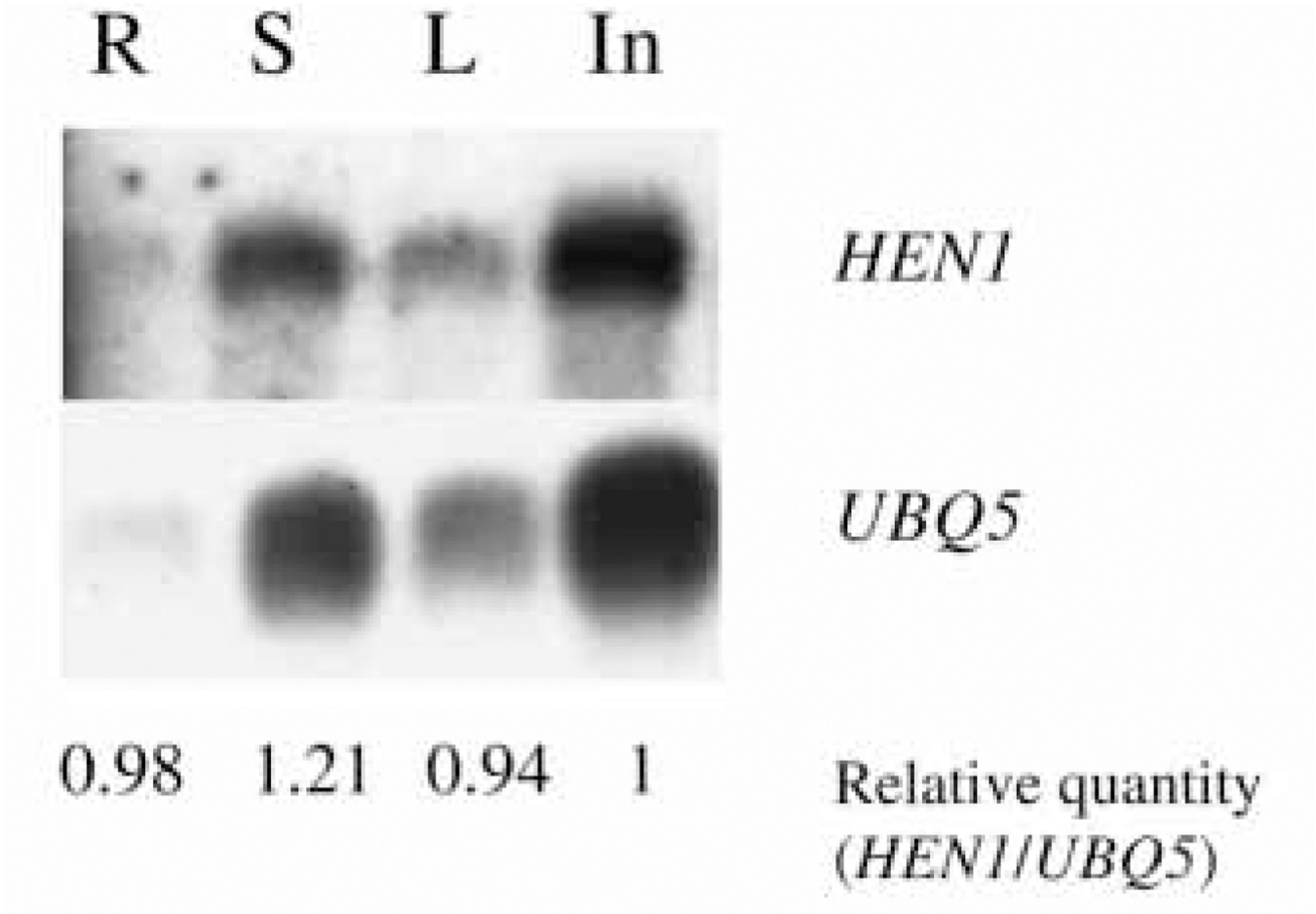

Fig. 7.

HEN1 RNA accumulation. *HEN1* RNA can be detected in roots (R), stems (S), leaves (L) and inflorescences (In). *UBQ5* was used as a loading control. The relative abundance of *HEN1* RNA in these tissues was calculated based on quantification with a phosphorimager.

Table 1

Rosette and cauline leaf numbers in various genotypes under long-day conditions

Genotype	Rosette leaf	Cauline leaf	*n*
L*er*	7.0±0.7	2.8±0.4	40
hen1-1	9.7±1.5	7.1±1.2	40
hen1-2	4.8±0.7	3.5±0.7	80
*hen1-1 T2**	10.2±0.9	3.9±0.6	20

Values are mean±standard deviation.

*T2 transgenic plants that are homozygous for *hen1-1* and either homozygous or hemizygous for the HEN1p10/p11 transgene.