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HUA ENHANCER2, a putative DExH-box RNA helicase, maintains homeotic B and C gene expression in *Arabidopsis*

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SUMMARY

Reproductive organ identity in *Arabidopsis* is controlled by the B, C and *SEPALLATA* classes of floral homeotic genes. We have identified a recessive mutation in a novel gene, *HUA ENHANCER2*, which, when combined with mutations in two weak class C genes, *HUA1* and *HUA2*, leads to the production of third whorl sepal-petal-stamens and fourth whorl sepal-carpels. Quadruple mutant analysis and in situ localization of A, B, C and *SEPALLATA* floral homeotic RNAs suggest that *HUA ENHANCER2* is required for the maintenance of B and C gene expression in the reproductive whorls. In addition to its role in floral homeotic gene expression, *HUA ENHANCER2* is required for normal spacing and number of perianth organ primordia. We show that *HUA ENHANCER2* encodes a putative DExH-box RNA helicase that is expressed in specific patterns in the inflorescence meristem and developing flowers. As a possible ortholog of the yeast exosome-associated protein, Dob1p (Mtr4p), HUA ENHANCER2 may affect floral organ spacing and identity through the regulation of protein synthesis or mRNA degradation. Therefore, our studies on *HUA ENHANCER2* not only demonstrate that B and C gene expression is established and maintained separately, but also implicate the existence of post-transcriptional mechanisms in the maintenance of B and C gene expression.

Key words: *Arabidopsis thaliana*, Flower, *HEN2*, Helicase, Stamen, Carpel

INTRODUCTION

Floral primordia initiate on the flanks of the *Arabidopsis* apical meristem in a regular, spiral phyllotaxy. Organ primordia then arise from the determinate floral meristem, commencing with four sepals, followed by four petals, six stamens and a terminal, bicarpellate gynoecium (Smyth et al., 1990). The organs initially arise as unspecified primordia, which then have their fates conferred upon them by the activities of four groups of genes. The classical ABC model describes the combinatorial interactions of three classes of homeotic genes (A, B and C) (Bowman et al., 1991a). Recent studies have demonstrated that the three redundant *SEPALLATA* genes *(SEP1-3)* are required for the function of the B and C genes in whorls 2, 3 and 4 (Pelaz et al., 2000). All of these genes, excepting *APETALA2* (*AP2*; class A), encode MADS-box containing transcription factors and are expressed in the whorls of the flower in which they act: class A gene *APETALA1* (*AP1*) in the first two whorls (Mandel et al., 1992), class B genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) in whorls 2 and 3 (Jack et al., 1992; Goto and Meyerowitz, 1994), class C gene *AGAMOUS* (*AG*) in whorls 3 and 4 (Yanofsky et al., 1990; Drews et al., 1991), and *SEP1-3* in whorls 2, 3, and 4 (Ma et al., 1991; Flanagan and Ma, 1994; Savidge et al., 1995; Mandel et al., 1998). *AP2* encodes a transcription factor containing an AP2-box and is expressed throughout the flower (Jofuku et al., 1994).

An important aspect of floral homeotic gene regulation is the establishment and maintenance of their very specific domains of expression (and function). A major player in the initial expression of *AP1, AP3, PI* and *AG* is the meristem identity gene *LEAFY (LFY)* (Schultz and Haughn, 1991; Weigel et al., 1992). *LFY* encodes a transcription factor that is expressed throughout young floral primordia and is required for the activation of all of these genes (Parcy et al., 1998). With the exception of *AP1*, whose initial floral expression pattern is the same as *LFY*, activation of floral organ identity genes by *LFY* requires a region-specific partner of *LFY*. *UNUSUAL FLORAL ORGANS*, which is localized first in the inner three and then in the second and third whorls, is needed to activate *AP3* and *PI* (Levin and Meyerowitz, 1995; Lee et al., 1997; Samach et al., 1999). The stem cell fate promoting gene *WUSCHEL (WUS)* is expressed in the center of the floral meristem and acts with *LFY* to activate *AG* (Lenhard et al., 2001; Lohmann et al., 2001). While *WUS* may be the specificity factor that promotes the initial expression of *AG* in the center of the flower, the repressing activity of the A gene *AP2* and the cadastral genes *LEUNIG, STERILE APETALA* and *AINTEGUMENTA* is necessary to prevent *AG* activation in the outer two whorls of the flower (Drews et al., 1991; Liu and Meyerowitz, 1995;

Byzova et al., 1999; Krizek et al., 2000). At the same time, the activation of *AG* leads to the loss of *AP1* expression from the innermost whorls of the flower (Gustafson-Brown et al., 1994). While there is much information on the activation and the restriction of homeotic gene expression, little is known about the maintenance of their expression in the developing floral organs. The continued expression of *AP3* and *PI* following their activation has been shown to be due, at least in part, to autoregulation by an AP3-PI complex that binds directly to the *AP3* promoter and acts indirectly on *PI* transcription (Jack et al., 1994; Hill et al., 1998; Tilly et al., 1998; Chen et al., 2000; Honma and Goto, 2000). The mechanism of *AP1* and *AG* maintenance, however, is not yet known.

While the initial screens for floral organ identity genes led to the isolation of multiple A and B function genes, only one C gene, *AG*, was isolated. A screen for mutations that enhanced the phenotypes of the weak *ag-4* allele led to the isolation of two genes, *HUA1* and *HUA2* (Chen and Meyerowitz, 1999). *HUA1* and *HUA2* appear to share *AG*'s functions in reproductive organ identity, repression of *AP1* gene expression, and determinacy. In this report we describe the use of an enhancer screen in the weak *hua1-1 hua2-1* double mutant background to isolate a mutation in a novel gene, *HUA ENHANCER2* (*HEN2*). Studies of the *hen2-1* mutation in the *hua1-1 hua2-1* background reveal a role of *HEN2* in the regulation of homeotic B and C gene expression. In addition, there is a defect in organ primordia initiation in *hen2-1* single mutants. *HEN2* was cloned through chromosome walking and was found to encode a putative DExH-box RNA helicase. Consistent with its proposed roles in flower development, *HEN2* is expressed in specific patterns in the inflorescence meristems and in developing flowers.

MATERIALS AND METHODS

Plant materials and growth conditions

The mutant strains used in this study, *hua1-1 hua2-1*, *hua1-1 hua2-1 ap1-1*, *hua1-1 hua2-1 ap2-2*, *hua1-1 hua2-1 ap3-3*, *hua1-1 hua2-1 pi-3* (Chen and Meyerowitz, 1999), *ap2-2*, *ag-3* (Bowman et al., 1991a) and *ag-4* (Sieburth et al., 1995) are in the Landsburg *erecta* (L*er*) ecotype. Plants were grown at 23°C in Pro-mix BX (Premier) under continuous light or 16-hour light/8-hour dark cycles.

To screen for enhancers of the *hua1-1 hua2-1* mutant phenotype, seeds were mutagenized by incubating in 0.2% EMS/0.01% Tween 20 for 12 hours. M_2 lines were collected from individual M_1 plants and screened for enhancers of the *hua1-1 hua2-1* phenotype. The DH380 line, once identified, was recovered from sibling plants and the background was cleaned by two backcrosses to *hua1-1 hua2-1*.

Positional cloning of *HEN2* and isolation of the *HEN2* cDNA

The mapping population was derived from a cross between *hen2-1/+ hua1-1 hua2-1* and wild-type Columbia. F_2 populations segregating *hen2-1* were identified, which were screened for plants of both the *hen2-1 hua1-1 hua2-1* and *hua1-1 hua2-1* phenotypes. Plants of the latter phenotype could be used for mapping as they could be genotyped for *hua1-1* and *hua2-1* using molecular markers (see below) and their *HEN2* genotype could be determined by looking for segregation of the triple mutant phenotype in the F_3 generation. DNA was prepared from these plants using the technique of Edwards et al. (Edwards et al., 1991). Forty *hen2-1 hua1-1 hua2-1* plants were used to map the *HEN2* locus to the top of chromosome 2 near the centromere between CAPS markers RNS1a and *PHYB*. Owing to difficulties using *PHYB*, an SSLP marker was designed on BAC F19G14 (F19G14p1/p2) approximately 5 cM to the north of *PHYB* and used along with RNS1a to screen for recombinants in 655 *hua1-1 hua2-1*-like plants. Plants showing recombination at these two markers were used for progressive fine mapping using SSLP and CAPS markers generated from sequence information provided by the *Arabidopsis* Genome Initiative and Cereon. Information on the CAPS and SSLP markers generated during this mapping is available at the Chen lab web site (http://waksman.rutgers.edu/~xuemei). Finally, 2 recombinants each remained at markers F18P14p7/p8 and T4E14p9/p10 but not at markers F18P14p5/p6 or T4E14p7/p8, respectively (see Fig. 5A). Four predicted genes from the 6 BACs in this interval were sequenced, including T4E14.10, in which a C to T substitution was found in the third exon, resulting in a proline to leucine change in the predicted protein.

5′ and 3′ RACE (Gibco-BRL) were employed to identify the 5′ and 3′ UTRs of *HEN2*, respectively, using primers designed from the predicted T4E14.10 sequence. Primers were then designed from the ends of the UTRs [T4E14p37 (5′-aaaacgacaaaaccccgttttcttcg-3′)/T4E14p38 (5′-tctgcaaatttcttcattatctaacttgttgg-3′)] to amplify the full-length cDNA from the 3′RACE first strand synthesis reaction using Ex-Taq (Panvera). The resulting 3.3 kb product was cloned into pGEM-T Easy (ProMega) and sequenced to give the exon-intron junctions of *HEN2* and to ensure the absence of mutations. The *HEN2* cDNA sequence is in GenBank under the accession number AY050658.

Identification of double mutants and construction of quadruple mutants and transgenic lines

hen2-1 hua1-1 and *hen2-1 hua2-1* plants were first identified in the mapping population when some *hua1-1 hua2-1*-like plants were found not to be *hua1-1 hua2-1* by molecular genotyping. Double mutants of *hen2-1* with *hua1-1* and *hua2-1* were then isolated from the F_2 of a backcross of *hen2/+ hua1-1 hua2-1* to L*er* by their 'twisty' silique and sterile *hua1-1 hua2-1*-like silique phenotypes, respectively. *hen2-1* plants were identified since they can have a 'mild' version of the *hen2-1 hua1-1* silique phenotype. The genotypes of these double and single mutants were confirmed by both backcrossing to *hua1-1 hua2-1* to show segregation of the *hen2-1 hua1-1 hua2-1* triple mutant phenotype and by genotyping for the loss of an *Aci*I site caused by the *hen2-1* mutation using T4E14p5 (above)/T4E14p20 (5′-cttcgaagacagtgtaaaagatgctatcc-3′).

Quadruple mutants containing *hen2-1*, *hua1-1*, *hua2-1* and *ag-4* were constructed using *ag-4* to pollinate *hua1-1 hua2-1 hen2-1/+* flowers. The resulting F_2 lines were planted to determine which were segregating the *hen2-1 hua1-1 hua2-1* triple mutant phenotype. F_2 plants with an enhanced *ag-4* phenotype were chosen from these lines and their genotypes were determined using molecular markers: *hua1-1*, MBK21p28a (5′-catgtaatgcctagacaatgagtatg- 3′)/MBK21p35a(5′-tttggagaaagctgcaggtttg-3′) cleaved with *Hph*I; *hua2-1*, MDK15p7-2 (5′-gctcctttgtcttattctca-3′)/MDK15p31 (5′-caaggtctgttgccattctg-3′) cut with *Pst*I; *hen2-1*, T4E14p50 (5′-gctcgtcctttccacgacgtccagc-3′)/T4E14p51 (5′-gctgtccatcattctggtcttcttcctgtc-3′) cleaved with *Aci*I; *ag-4*, AGp16 (5′-aagtattacccgaatccgatccaagaa-3′)/AGp17 (5′-tagtcga-tttcagaaaataagagcagatt-3′) cut with *Alw*NI.

hen2-1 hua1-1 hua2-1 ap2-2 quadruple mutants were constructed by pollinating a *hen2-1/+ hua1-1 hua2-1* plant with *ap2-2* pollen. F_1 plants heterozygous for *hen2-1* were identified and the corresponding F_2 lines were screened for plants with small flowers with *hen2-1 hua1-1 hua2-1* gynoecia that lacked normal sepals. The genotype was confirmed using molecular markers (see above).

hen2-1 hua1-1 hua2-1 quadruple mutants with a*p1-1*, *ap3-3* and *pi-3* were created by crossing *hen2-1/+ hua1-1 hua2-1* plants with *hua1-1 hua2-1 ap1-1*, *hua1-1 hua2-1 ap3-3* and *hua1-1 hua2-1 pi-3* plants, respectively. *hen2-1 hua1-1 hua2-1 ap1-1* quadruple mutants in the F_2 were identified by their small flower size and the characteristic

secondary flowers in the axils of the first whorl organs. Their *hen2-1* genotype was confirmed by molecular genotyping. *hen2-1 hua1-1 hua2-1 ap3-3* and *hen2-1 hua1-1 hua2-1 pi-3* quadruple mutants were identified by their small size, sepals in the second whorl and *hen2-1 hua1-1 hua2-1*-type gynoecia, and confirmed by molecular genotyping.

A *HEN2* genomic clone (HEN2g) was generated by cloning an 11.1 kb *Sal*I/*Eco*RI fragment including the whole T4E14.10 coding region plus 3.5 kb upstream and 1.5 kb downstream sequences from BAC T4E14 (provided by the *Arabidopsis* Biological Resource Center) into the plant transformation vector pPZP211 (Hajdukiewicz et al., 1994). A mutant version of HEN2g (mHEN2g) was created using site-directed mutagenesis to recreate the mutation found in *hen2-1*. A 4.8 kb *Bam*HI fragment of HEN2g was subcloned into pBluescript SK+ (Stratagene) and PCR was performed using *Pfu* polymerase (Stratagene) and mutagenesis primers T4E14p35 (5′-gggtttgaatatg-ctcgccaaaactgtag-3′) and T4E14p36 (5′-catcagttttggcgagcatattca-aaccc-3′). The resulting product was then incubated with *Dpn*I to digest the methylated plasmid template. The mutagenized subclone was sequenced to confirm the presence of the mutation and the lack of unwanted PCR-induced mutations and a *Sfo*I/*Sac*I fragment was cloned into HEN2g to create mHEN2g.

hen2-1 hua1-1 plants and *hen2-1 hua1-1* populations segregating *hua2-1* were transformed with HEN2g, mHEN2g and the empty vector pPZP211 using the vacuum infiltration method (Bechtold et al., 1993). Transgenic seedlings were selected on AT medium (Haughn and Somerville, 1986) containing 50 μg/ml kanamycin and transferred to soil.

Floral phenotypes were recorded using a Fuji digital camera HC300-Z and manipulated using Adobe Photoshop.

RNA filter hybridization, in situ hybridization and scanning electron microscopy (SEM)

RNA filter hybridization was carried out as described (Li et al., 2001). In situ hybridization with digoxigenin (DIG)-labeled probes was carried out according to the protocol given at www.wisc.edu/genetics/CATG/barton/protocols.html, except that UTP and DIG-UTP were used at a 3:7 ratio in probe synthesis. *AP1* (Gustafson-Brown et al., 1994), *AP3* (Jack et al., 1992), *PI* (Goto et al., 1994), *AG* (Drews et al., 1991), *SEP1* (Ma et al., 1991), *SEP2* (Savidge et al., 1995) and *SEP3* (Mandel and Yanofsky, 1998) antisense templates were prepared as previously described. *HEN2* antisense and sense probes were created using PCR to amplify an 838 bp fragment of the *HEN2* cDNA (T4E14p43 [5′-cttcagttggaactggttccgc-3′]/T4E14p46 [5′-tccacatactcttccacgtcaatttccag-3′]), which was cloned into pCR-BluntII-TOPO (Invitrogen). The antisense probe was made by linearizing with *Bam*HI and transcribing with T7 RNA polymerase, while the sense probe was synthesized by digestion with *Xba*I and transcribing with SP6 polymerase. Results were analyzed using differential-interference contrast optics on a Zeiss Axioplan and images were manipulated using Adobe Photoshop.

Fixation for SEM was performed as previously described (Modrusan et al., 1994) and dehydrated samples were dried in a Balzers CPD020. Dissected samples were coated with gold-palladium using a Balzers SCD004 sputter coater and viewed in an Amray SEM 1830I under accelerating voltage of 20 kV. Digital images were captured and manipulated in Adobe Photoshop.

RESULTS

Isolation of the *hen2-1* mutant

In order to identify mutations in novel genes acting in the C pathway of floral organ development, a sensitized screen was undertaken through mutagenesis of the weak *hua1-1 hua2-1* double mutant. *hua1-1 hua2-1* double mutant flowers are marked by occasional petaloid stamens in the third whorl of early arising flowers and by a consistent broadening at the top of the gynoecium (compare Fig. 1A with 1B and 1N with 1O) (Chen and Meyerowitz, 1999). M_2 lines from an ethyl methanesulfonate mutagenesis were screened for enhancement of the reproductive organ identity defects. Amongst the several enhanced lines isolated, one (DH380) had flat, green and/or white organs in the third whorl in the place of stamens and a small, shrunken gynoecium (Fig. 1C,P).

Backcrosses to *hua1-1 hua2-1* double mutants demonstrated that DH380 contained one additional, recessive mutation. Mapping to just north of the centromere of chromosome 2 (see below) demonstrated that the mutation was not in *HUA1*, *HUA2*, *AG* or any other known floral homeotic gene. Thus, we have identified a novel gene affecting reproductive organ identity, which we have named *HUA ENHANCER2 (HEN2)*. Throughout the remainder of this paper, DH380 will be referred to by its genotype: *hen2-1 hua1-1 hua2-1*.

Vegetative and inflorescence phenotype of *hen2-1*

hen2-1 single mutants were isolated from a backcross to wild-type L*er*. There appear to be no gross defects in plant height, inflorescence number, leaf number or size between *hen2-1* and wild-type plants (data not shown). No examination was made of the root phenotype. While both *hua1-1* and *hua2-1* plants appear wild-type in terms of vegetative growth, *hua1-1 hua2-1* plants are shorter than wild-type plants and have fewer and smaller leaves (Chen and Meyerowitz, 1999; data not shown). The addition of *hen2-1* led to a further reduction in height (compare Fig. 1G with 1D-F). These results suggest that *HEN2* may play redundant roles with *HUA1* and *HUA2* in stem elongation.

While there are no obvious changes in basic plant growth in the *hen2-1*single mutant, there appear to be alterations in production of flowers along the inflorescence. When *hen2-1* inflorescences are viewed from the side, the spacing between siliques appears to be compressed and phyllotaxy can be disrupted (compare Fig. 1D and 1E). When *hen2-1* inflorescences are viewed from above, there are a larger number of mature flowers and buds than in L*er* inflorescences (Fig. 1H,I). Similar changes in both flower number and phyllotaxy are seen in double and triple mutants of *hen2-1* with *hua1-1* and *hua2-1* (Fig. 1F,G; data not shown), but not in *hua1-1*, *hua2-1*, or *hua1-1 hua2-1*(data not shown). SEM of the inflorescence meristems of *hen2-1* plants reveals that there is no gross increase either in the size of the inflorescence meristem or in the number of floral primordia surrounding the inflorescence meristem (Fig. 2A,B). It remains to be determined whether the rates of floral primordia initiation and subsequent differentiation are increased in *hen2-1*.

hen2-1 affects floral organ number and positioning

While *hen2-1* single mutants appear to be unaffected in floral organ identity (Fig. 1J), they are affected in other aspects of flower development. From a distance, *hen2-1* inflorescences look disorganized and the buds appear to open earlier than those in wild-type inflorescences. Closer examination reveals that this is due to two changes in flower architecture. First, the number and positioning of the perianth organs are extremely variable. In a significant proportion of these flowers, the number of sepals is altered and can vary from two to six

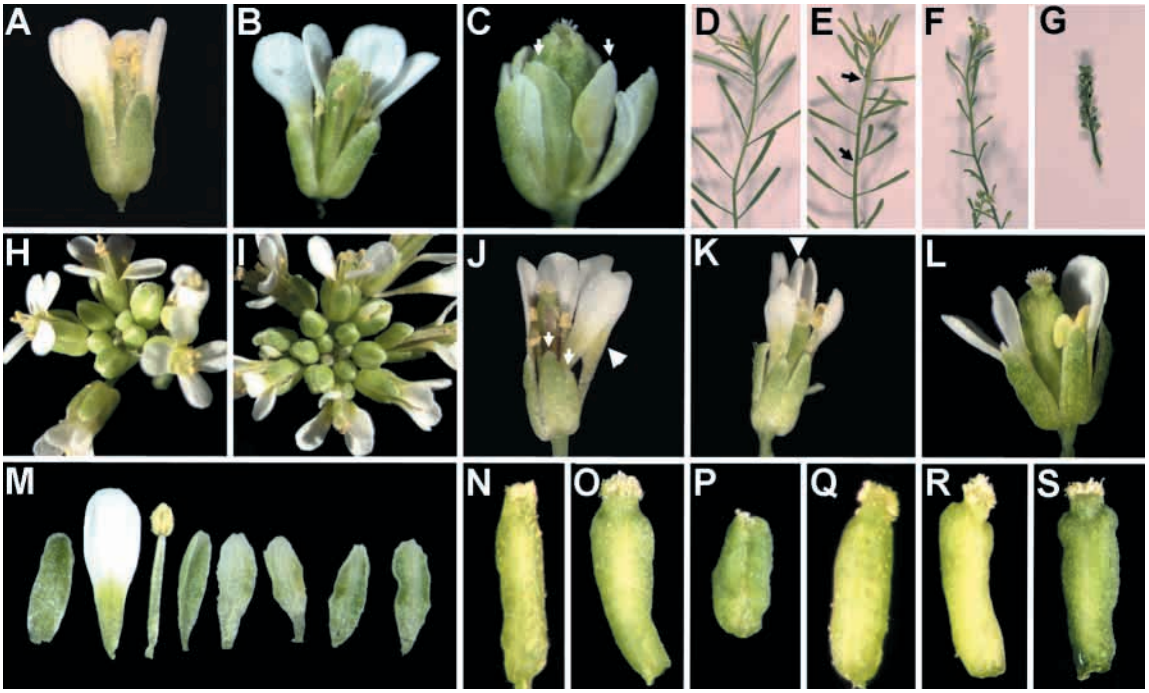

Fig. 1. Inflorescence and floral phenotypes of *hen2-1* mutants. (A) L*er*; (B) *hua1-1 hua2-1*; (C) *hen2-1 hua1-1 hua2-1*, petals are small and third whorl organs are a mosaic of sepal-petal tissue (arrows). (D-G) Side view of inflorescences, all at same magnification. (D) L*er*. (E) *hen2-1*, note increased number of siliques and altered phyllotaxy (arrows). (F) *hua1-1 hua2-1*. (G) *hen2-1 hua1-1 hua2-1*, note reduction in internode length. (H) L*er* inflorescence. (I) *hen2-1* inflorescence, note the increased number of flowers and buds. (J) *hen2-1* flower, note crowding of a lateral sepal with a medial sepal (arrows) and an extra petal (arrowhead). (K) *hen2-1 hua1-1* flower, exhibiting an extra petal (arrowhead). (L) *hen2-1 hua2-1* flower, note the short, apparently immature stamens. (M) Comparison of *hen2-1 hua1-1 hua2-1* third whorl organs (five organs on the right), with sepal, petal and stamen from a *hua1-1 hua2-1* flower. (N-S) Isolated gynoecia. (N) L*er*. (O) *hua1-1 hua2-1*, note broadening at top of gynoecium. (P) *hen2-1 hua1-1 hua2-1*, gynoecium is small and bulging, with reduced stigmatic papillae. (Q) *hen2-1*, note lack of bulge. (R) *hen2-1 hua1-1*, the gynoecium bulges at the top. (S) *hen2-1 hua2-1*, note broadening at top.

(compare Fig. 2C and 2D). The sepals can be larger than normal or be narrower or even reduced to almost a filament. In addition, the regular, even spacing is altered, with the lateral sepals often crowding against the medial sepals (Fig. 1J; Fig. 2B,D). Petal number also can be affected (Fig. 1J). In many cases, the number and spacing of petals are adjusted such that a petal is found between two sepals; other times, the normal number and positioning are preserved despite the changes to the sepal whorl. Occasionally, sepal-petal mosaic organs are found (data not shown). The second contributor to the disorganized appearance is growth of organs, usually petals, at odd angles out of the flower (data not shown). Similar changes are also seen in *hen2-1 hua1-1* flowers (Fig. 1K). In both *hen2-1 hua2-1* and *hen2-1 hua1-1 hua2-1* flowers, however, there are only reductions in the number of sepals and/or petals (data not shown).

hen2-1 affects organ identity in the reproductive whorls

A detailed examination of *hen2-1 hua1-1 hua2-1* triple mutant flowers revealed that they are affected in several aspects of flower development (Fig. 1C). The triple mutant flowers are approximately one third to one half the size of wild-type flowers (data not shown). This appears to be largely due to a reduction in size of the organs in the inner three whorls of the flower. While the sepals appear to be normal, the petals are much reduced in size, only occasionally growing larger than the sepals. The most severe changes occur in the third whorl organs, which appear as small, flat organs that can resemble sepals, sepal-petals, petals or petal-stamens (Fig. 1M).

Scanning electron microscopy (SEM) was used to investigate the identity of these organs at the cellular level (Fig. 2). Each organ in the *Arabidopsis* flower has stereotypical external cell features that enable identification of cell type (Kunst et al., 1989; Smyth et al., 1990). Abaxial sepal cells are irregular in shape and have marked epicuticular striations. Interspersed with these are occasional, extremely long cells (Fig. 2E). Petal cells are small and pebble-like. Those on the adaxial face of the organ are cone-shaped with stellate epicuticular thickenings radiating from the tip (Fig. 2F), while those on the abaxial side are flat with less regular thickenings (Fig. 2G). The cells of anthers, conversely, resemble jigsaw puzzle pieces (Fig. 2H) and valve cells of the ovary are irregular in shape and lack epicuticular thickenings (Fig. 2L). Examination of the third whorl organs of *hen2-1 hua1-1 hua2-1* flowers confirmed at the cellular level the mosaicism suggested by their gross morphological appearance. Mixtures of round, petal-like cells and irregular cells with thickenings reminiscent of sepal cells or jigsaw puzzle-like stamen cells were consistently found on the surface of these third whorl organs (Fig. 2I-K). In addition, a study of the ontogeny of *hen2-1 hua1-1 hua2-1* flowers showed that third whorl organ identity becomes aberrant very early in development. During flower development in *Arabidopsis*, the stamens first arise as

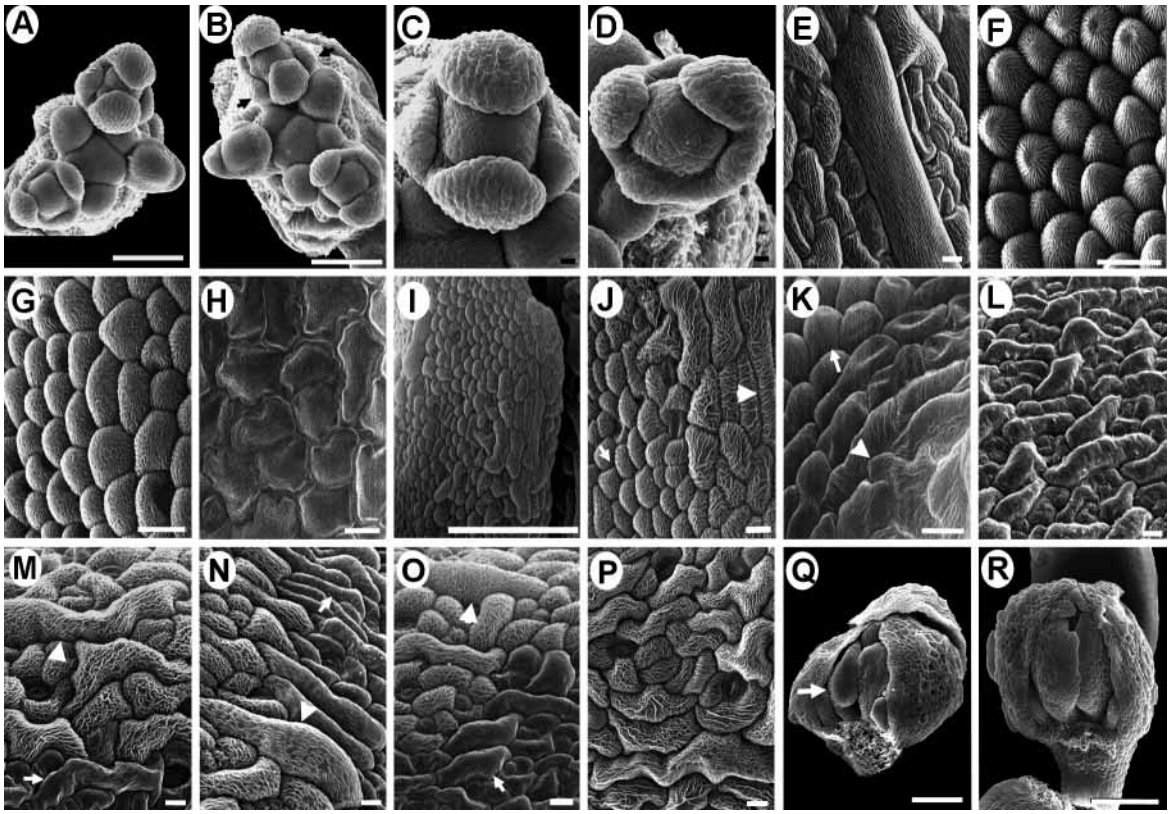

Fig. 2. Scanning electron micrographs of mature and developing flowers of wild-type and *hen2-1* single, and double and triple mutants with *hua1-1* and *hua2-1*. (A) L*er* inflorescence meristem. (B) *hen2-1* inflorescence meristem, note crowding of lateral sepals towards the adaxial sepal in top developing flower (arrows). (C) L*er* at stage 4-5 with four evenly spaced sepal primordia. (D) *hen2-1* at stage 4-5, note extra sepals and disrupted spacing of sepals. (E) L*er* abaxial sepal cells, note epicuticular striations and the long cell. (F) L*er* adaxial petal cells. (G) L*er* abaxial petal cells. (H) L*er* stamen cells. (I) A *hen2-1 hua1-1 hua2-1* third whorl organ. (J) Higher magnification of part of I, showing patches of abaxial petal (arrow) and sepal-like cells (arrowhead). (K) Detail of a *hen2-1 hua1-1 hua2-1* third whorl organ showing both petal (arrow) and stamen-like cell types (arrowhead). (L) L*er* ovary valve cells. (M) The top, lateral region of a *hua1-1 hua2-1* ovary showing presence of ovary (arrow) and sepal-like cells (arrowhead). (N) The top, lateral area of a *hen2-1 hua1-1* ovary, note cells with both ovary (arrow) and sepal characteristics (arrowhead). (O) The top, lateral portion of a *hen2-1 hua2-1* ovary with both ovary (arrow) and sepal-like cells (arrowhead). (P) *hen2-1 hua1-1 hua2-1* ovary cells, all have sepal-like features. (Q) *hua1-1 hua2-1* at stage 7-8, note stalk of developing stamen (arrow). (R) *hen2-1 hua1-1 hua2-1* at stage 7-8 showing large, flat third whorl organs. Scale bars, 10 μm (C,D,E-H,J-P), 50 μm (I), 100 μm (A,B,Q,R).

undifferentiated, ellipsoid primordia. The first sign of differentiation is the appearance of a stalk at the base, creating a spade-shaped organ (Fig. 2Q) (Smyth et al., 1990). In *hen2-1 hua1-1 hua2-1* developing flowers, the third whorl organs at this stage are flat, resembling sepals or petals (Fig. 2R). These results suggest that the identity of the third whorl organs is disrupted not only before the differentiation of the epidermal cells, but also prior to differentiation at the whole organ level.

hen2-1 also results in carpel-to-sepal transformation in the *hua1-1 hua2-1* background. *hua1-1 hua2-1* gynoecia are broadened at the top relative to wild-type gynoecia (compare Fig. 1O with 1N). Examination using SEM revealed that this broadening is correlated with the appearance of cells with sepal-like striations and even occasional long cells (Fig. 2M). Thus, *hua1-1 hua2-1* gynoecia are mosaics of sepal and carpel cells, with the sepal features localized to the top, lateral edges of the gynoecium. The gynoecia of *hen2-1 hua1-1 hua2-1* are small and bulging, with reduced style and stigmatic tissue (Fig. 1P). Occasionally they are split open at the top and can be trilocular. In addition, they are sterile: when dissected, only a few aberrant ovules are found (data not shown). Examination by SEM showed that all ovary epidermal cells of *hen2-1 hua1-1 hua2-1* gynoecia had sepal-like epicuticular thickenings (Fig. 2P). Occasionally, straight trichomes, as normally seen on wild-type sepals, but never on gynoecia, also were found (data not shown). These data suggest that the conversion of the carpels towards sepal fate seen in the *hua1-1 hua2-1* double mutants was enhanced by the *hen2-1* mutation.

While both *hen2-1* and *hua1-1* flowers are wild-type in terms of organ identity (Fig. 1J) (Chen and Meyerowitz, 1999), *hen2-1 hua1-1* gynoecia are similar to those of *hua1-1 hua2-1* double mutants. They are broad at the top (Fig. 1R) and SEM analysis revealed the appearance of sepal-like cells on the lateral top edges of the ovaries (Fig. 2N). However, the silique phenotype differs slightly from *hua1-1 hua2-1*: *hua1-1 hua2-1* siliques are shortened and broad at the top, while *hen2-1 hua1-1* siliques are broad at the top and are often curved, giving a ‘twisty’ appearance (data not shown). The *hua2-1* phenotype is also enhanced by *hen2-1*. While *hua2-1* flowers are essentially wild-type, *hen2-1 hua2-1* double mutants are male sterile, having greenish, immature-appearing stamens that fail to elongate to full size or produce pollen (Fig. 1L). The

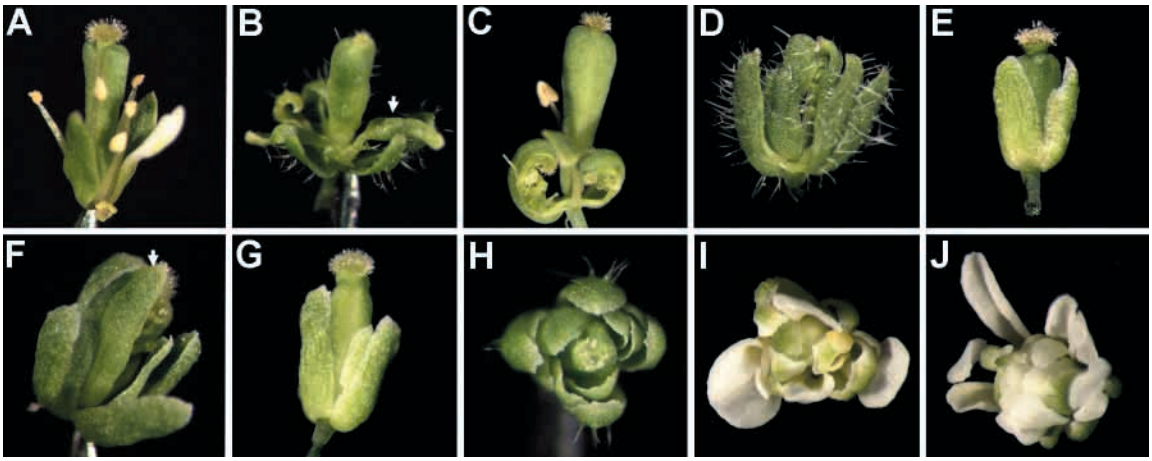

Fig. 3. Floral phenotypes of *hua1-1 hua2-1* triple mutants and *hen2-1 hua1-1 hua2-1* quadruple mutants with A, B and C class mutations. (A) *hua1-1 hua2-1 ap1-1*. (B) *hen2-1 hua1-1 hua2-1 ap1-1*, note stamens replaced by green organs (arrow). (C) *hua1-1 hua2-1 ap2-2*. (D) *hen2-1 hua1-1 hua2-1 ap2-2* with leafy organs in all whorls. (E) *hua1-1 hua2-1 ap3-3*. (F) *hen2-1 hua1-1 hua2-1 ap3-3* with third whorl sepals (arrow). (G) *hua1-1 hua2-1 pi-3*. (H) *hen2-1 hua1-1 hua2-1 pi-3* with third whorl sepals. (I) *hua1-1 hua2-1 ag-4*, note third whorl petals/petal-stamens and internal flower. (J) *hen2-1 hua1-1 hua2-1 ag-4*, the third whorl organs are narrow petals.

gynoecia of *hen2-1 hua2-1* flowers also resemble those of *hua1-1 hua2-1* flowers with respect to shape (Fig. 1S) and the appearance of sepal-like cells on the lateral edges (Fig. 2O). Normal ovary cells are found on *hen2-1* single mutant gynoecia (data not shown).

Phenotypes of quadruple mutants of *hen2-1 hua1-1 hua2-1* with other floral homeotic mutations

In order to further investigate the origin of the floral organ identity defects of *hen2-1 hua1-1 hua2-1* mutants, quadruple mutants were constructed with mutations in A, B and C genes (Fig. 3).

Class A: *hen2-1 hua1-1 hua2-1 ap1-1* and *hen2-1 hua1-1 hua2-1 ap2-2*

A strong mutant allele of *AP1*, *ap1-1*, leads to loss of petals, conversion of sepals to leaves and the production of secondary flowers in the axils of the first whorl leaves (Irish and Sussex, 1990). In a *hua1-1 hua2-1* background, the introduction of *ap1-1* leads to flowers largely resembling *ap1-1* in organ identity, although the broadening at the top of the gynoecium is somewhat repressed by the loss of *AP1* function (Fig. 3A) (Chen and Meyerowitz, 1999). Additional loss of *HEN2* leads to the production of green, sepal- or leaf-like third whorl organs and a bulging gynoecium with straight trichomes (Fig. 3B).

A strong mutant allele of *AP2*, *ap2-2* produces flowers with carpel-like organs in the first whorl, bearing stigmatic papillae and ovules (Bowman et al., 1991a). The second and third whorl organs are largely suppressed, but are stamens when present. The gynoecium can be either normal in appearance or consist of un- or partially-fused carpels. A combination of *ap2-2* with *hua1-1 hua2-1* is largely additive, with *ap2-2*-like flowers bearing *hua1-1 hua2-1* gynoecia and some reduction of carpel features in the first whorl organs (Fig. 3C) (Chen and Meyerowitz, 1999). *hen2-1 hua1-1 hua2-1 ap2-2* flowers have leaf-like, trichome-bearing organs in the first, second and third whorls, and gynoecia with unfused and often trichome-bearing organs with little stigmatic tissue (Fig. 3D). The suppression of the *ap2-2* outer whorl carpelloidy and loss of stamens from both A mutants demonstrate a reduction in C function in the quadruple mutants. In addition, the strong leafy character of the third whorl organs suggests reduction of B function in the third whorl.

Class B: *hen2-1 hua1-1 hua2-1 ap3-3* and *hen2-1 hua1-1 hua2-1 pi-3*

The strong *ap3-3* allele of the B gene *AP3* leads to the production of sepals in the second whorl and carpels or filaments that fuse to the fourth whorl carpels in the third whorl (Jack et al., 1992). The addition of the *hua1-1* and *hua2-1* mutations to an *ap3-3* background leads to an additive phenotype, whereby *ap3-3*-like flowers have bulges at the top of their gynoecia (Fig. 3E) (Chen and Meyerowitz, 1999). *pi-3* is a weak allele of the B gene *PI*, which results in sepals in the place of petals and free-standing carpel-like organs in the third whorl (Bowman et al., 1991a). *hua1-1* and *hua2-1* enhance the *pi-3* phenotype such that *hua1-1 hua2-1 pi-3* resembles *hua1-1 hua2-1 ap3-3*: the third whorl carpels are transformed into filaments and the gynoecium bulges at the top (Fig. 3G) (Chen and Meyerowitz, 1999). *hen2-1 hua1-1 hua2-1 ap3-3* and *hen2-1 hua1-1 hua2-1 pi-3* quadruple mutant flowers both produce sepals in the first three whorls and *hen2-1 hua1-1 hua2-1*-like gynoecia in the fourth whorl (Fig. 3F,H). The production of unfused sepals, rather than carpels, in the third whorl suggests reduction of C function in the third whorl. The fact that the weak *pi-3* allele resembles the strong *ap3-3* allele in producing sepals in the third whorl in the *hen2-1 hua1-1 hua2-1* background suggests that B function is also compromised in the third whorl in this background.

Class C: *hen2-1 hua1-1 hua2-1 ag-4*

Strong mutant alleles of *AG*, such as *ag-3*, lead to stamens replaced with petals and internal flowers in the place of a gynoecium (Bowman et al., 1991a). *hua1-1* and *hua2-1* were identified through their enhancement of the weak *ag* allele, *ag-*

4, which is normal in the first three whorls, but has internal flowers (Sieburth et al., 1995). A triple mutant of *hua1-1 hua2-1 ag-4* was described as resembling strong *ag* alleles (Chen and Meyerowitz, 1999). However, under our growth conditions, third whorl organs of *hua1-1 hua2-1 ag-4* flowers include both petal-stamens and petals (Fig. 3I). Conversely, quadruple mutants of *hen2-1 hua1-1 hua2-1 ag-4* grown under the same conditions have petals in the third whorl (Fig. 3J), resembling *ag-3* mutants and suggesting enhancement of the *hua1-1 hua2-1 ag-4* phenotype due to further loss of C function in the third whorl. However, these quadruple mutant flowers are not completely identical to *ag-3* flowers: the petals are narrower.

Floral organ identity gene expression in *hen2-1 hua1-1 hua2-1* mutants

In addition to studying quadruple mutants, interactions between *HEN2* and the floral homeotic genes were investigated using in situ localization of A, B, C and *SEP* mRNAs in *hen2-1 hua1-1 hua2-1* versus *hua1-1 hua2-1* flowers (Fig. 4).

AP1

AP1 mRNA is detected at the earliest stages throughout the floral primordium. However, starting at early stage 3, the expression of *AG* leads to its restriction to the initiating sepals and the presumptive second whorl. As the petals and sepals develop, *AP1* continues to be expressed in those organs (Mandel et al., 1992; Gustafson-Brown et al., 1994). The early expression of *AP1* is normal in both *hen2-1 hua1-1 hua2-1* (Fig. 4A) and *hua1-1 hua2-1* (data not shown). After stage 6, unlike wild-type flowers, low level of *AP1* expression is detected in patches of both the stamens and carpels of *hua1-1 hua2-1* flowers (Fig. 4C) (Chen and Meyerowitz, 1999). This ectopic expression is greatly enhanced in *hen2-1 hua1-1 hua2-1* triple mutants, with the patches of *AP1* transcripts in developing third and fourth whorl organs approaching the level seen in the outer two whorls (Fig. 4B).

AP3 and PI

AP3 mRNA is first seen at early stage 3 in two patches on either side of the floral meristem, predicting the location of the second and third whorls. It is later found in the initiating petal and stamen primordia and continues to be expressed in these organs as they develop (Jack et al., 1992). *PI* expression is identical to that of its partner, *AP3*, with the exception that it is first initiated in the inner three whorls of the floral primordium and shortly thereafter resolves to the second and third whorls (Goto and Meyerowitz, 1994). The early expression patterns of both *AP3* and *PI* are unchanged in *hen2-1 hua1-1 hua2-1* (Fig. 4D,G) and *hua1-1 hua2-1* (data not shown). However, while their expression continues normally in the second and third whorl organs of *hua1-1 hua2-1* (Fig. 4F,I), the expression of both becomes patchy in the third whorl of *hen2-1 hua1-1 hua2-1* after stage 6. They are found only in parts of some organs and are absent from others (Fig. 4E,H).

AG

AG is transcribed initially at early stage 3 in the central dome of the floral meristem, marking the future location of the reproductive whorls (Yanofsky et al., 1990; Drews et al., 1991). As the reproductive organs arise, *AG* continues to be expressed

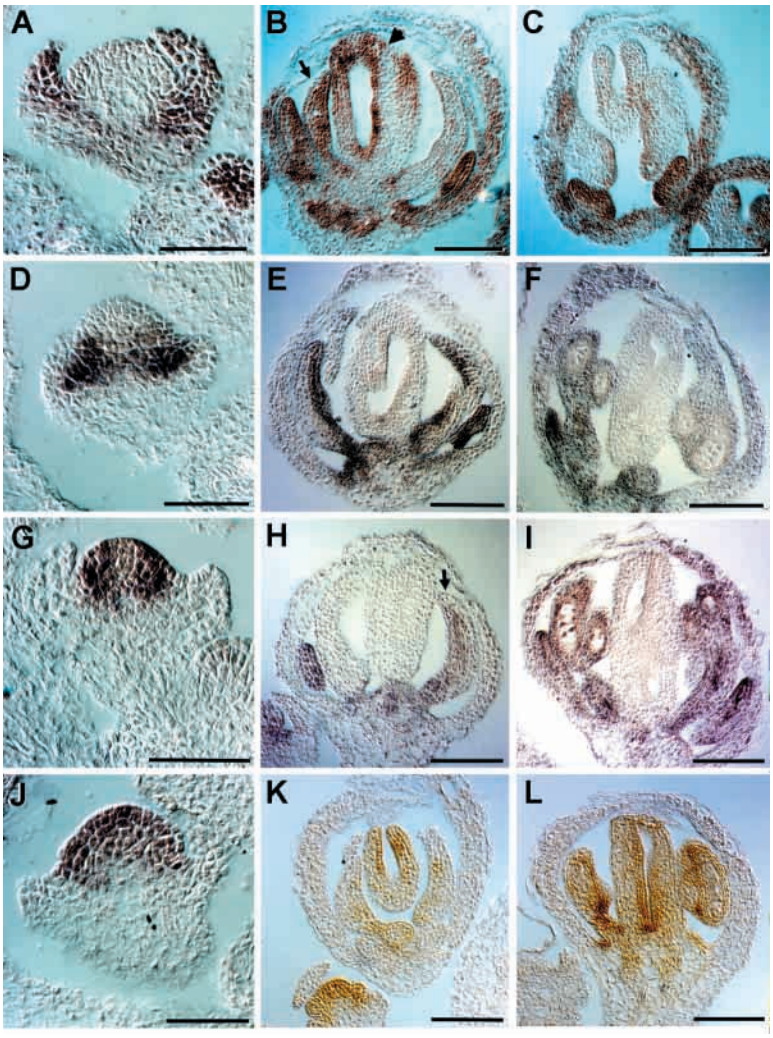

Fig. 4. In situ localization of RNAs from floral homeotic genes in *hen2-1 hua1-1 hua2-1* and *hua1-1 hua2-1* developing flowers. (A-C) Hybridization with *AP1* antisense probe. (A) *hen2-1 hua1-1 hua2-1* at stage 4. (B) *hen2-1 hua1-1 hua2-1* at stage 8-9 showing strong patches of *AP1* expression in the gynoecium (arrowhead) and one third whorl organ (arrow). (C) Stage 8-9 *hua1-1 hua2-1* from the same slide as (B) with weak expression of *AP1* in the third and fourth whorls. (D-F) Hybridization with *AP3* antisense probe. (D) Stage 3 *hen2-1 hua1-1 hua2-1*. (E) Stage 8-9 *hen2-1 hua1-1 hua2-1* with patchy *AP3* expression in the third whorl organs. (F) *hua1-1 hua2-1* at stage 8-9. (G-I) Hybridization with *PI* antisense probe. (G) Stage 3 *hen2-1 hua1-1 hua2-1*. (H) *hen2-1 hua1-1 hua2-1* at stage 8-9, note *PI* expression in only one of the visible third whorl organs (arrow). (I) Stage 8-9 *hua1-1 hua2-1*. (J-L) Hybridization with *AG* antisense probe. (J) Stage 3 *hen2-1 hua1-1 hua2-1*. (K) *hen2-1 hua1-1 hua2-1* at stage 8 with low expression in the third and fourth whorl organs, note strong expression of *AG* in a stage 5 flower at the bottom of the panel. (L) Stage 8 *hua1-1 hua2-1* flower from the same slide as (K). Scale bars, 50 μm (A,D,G,J), 100 μm (B,C,E,F,H,I,K,L).

throughout them, finally being resolved to the tapetum of the stamens and the stigmatic region and ovules of the gynoecium (Bowman et al., 1991b; Drews et al., 1991). The early pattern of *AG* expression is normal in both *hen2-1 hua1-1 hua2-1* (Fig. 4J) and *hua1-1 hua2-1* (data not shown), and continues to be so for *hua1-1 hua2-1* flowers (Fig. 4L). However, the level of *AG* expression appears to decrease after stage 6 in *hen2-1 hua1-1 hua2-1* flowers, especially in the third whorl organs, where *AG* mRNA can be present in only portions of the organs (Fig. 4K).

SEP1, 2, 3

SEP1, 2, 3 are all expressed in the three inner whorls of the flowers starting just before B and C gene expression and continuing throughout development. In addition, *SEP1* and *SEP2* are expressed in the first whorl of young flowers (Ma et al., 1991; Flanagan and Ma, 1994; Savidge et al., 1995; Mandel et al., 1998). The expression of all three *SEP* genes is normal in both *hua1-1 hua2-1* and *hen2-1 hua1-1 hua2-1* developing flowers (data not shown).

Molecular cloning of *HEN2*

In order to map the *HEN2* locus, *hen2-1/+ hua1-1 hua2-1* plants in the L*er* ecotype were crossed to wild-type plants of the Columbia ecotype. Forty F_2 plants with the *hen2-1 hua1-1 hua2-1* phenotype were used to map *HEN2* to the top of chromosome 2 between CAPS markers RNS1a and *PHYB*. A further 655 plants were used to fine map *HEN2* using CAPS and SSLP markers designed from sequences generated by the *Arabidopsis* Genome Initiative and the Cereon SNP collection.

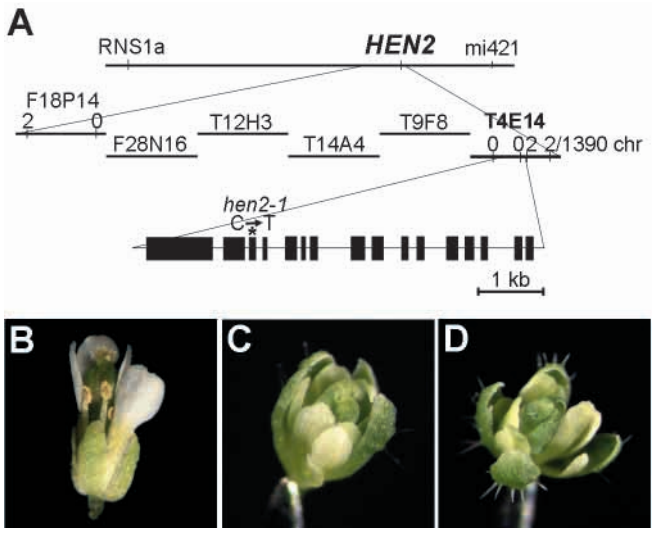

Fig. 5. Cloning of *HEN2*. (A) Mapping of the *HEN2* locus to six BACs at the top of chromosome 2. Numbers represent the number of recombinant chromosomes in a total of 1390 chromosomes. The schematic below shows the basic structure of *HEN2* with exons and introns represented by boxes and lines, respectively. The nucleotide substitution in exon 3 found in *hen2-1* is marked. (B-D) Molecular complementation of *hen2-1 hua1-1 hua2-1*. (B) A flower from a *hen2-1 hua1-1 hua2-1* plant containing HEN2g, showing rescue of the third whorl and gynoecium phenotypes. (C) A flower from a *hen2-1 hua1-1 hua2-1* plant containing the vector alone. (D) A flower from a *hen2-1 hua1-1 hua2-1* plant containing mHEN2g. Note lack of rescue in C and D.

The *HEN2* locus was narrowed down to a region of 6 BACs just north of the centromere marker mi421 (Fig. 5A).

This region, owing to its proximity to the centromere, is rich in repetitive DNA and contains few genes (Copenhaver et al., 1999). Thus, to identify the *HEN2* locus, we sequenced four genes from these BACs in *hen2-1*, finding a C to T nucleotide substitution in a predicted exon of T4E14.10 (Fig. 5A). 5′ and 3′ RACE were used on total inflorescence RNA to identify the 5′ and 3′ ends of the *HEN2* RNA and primers designed from these ends were used to amplify a 3348 bp *HEN2* cDNA. This cDNA varied only in three exon-intron junctions from the protein coding region predicted by the *Arabidopsis* Genome Initiative (Accession number At2g06990). The nucleotide substitution in *hen2-1* leads to the replacement of a proline with a leucine.

In order to confirm the identity of T4E14.10 as *HEN2*, we cloned an 11.1 kb region of T4E14 containing the entire *HEN2* coding region plus 3.5 kb upstream and 1.5 kb downstream sequences into the plant transformation vector pPZP211. No other predicted genes are found in this fragment. This construct, HEN2g, was used to transform a population homozygous for *hen2-1* and *hua1-1* and segregating for *hua2-1*. Three triple mutant plants containing the construct were identified through molecular genotyping of *hua2-1* and all had flowers resembling *hua1-1 hua2-1* double mutants (Fig. 5B). No rescue was seen in plants containing only the vector (Fig. 5C). HEN2g was also transformed into *hen2-1 hua1-1* plants and rescued both the increased flower number and the ‘twisty’ silique phenotype, while the vector alone did not (data not shown). In addition, to further confirm the identity of T4E14.10 as *HEN2*, the *hen2-1* mutation was re-created in the genomic clone using site-directed mutagenesis. This construct, mHEN2g, was unable to rescue either the triple or *hen2-1 hua1-1* mutant phenotypes (Fig. 5D; data not shown).

HEN2 encodes a putative DExH-box RNA helicase

The full length cDNA of *HEN2* encodes a predicted protein of 991 amino acids (Fig. 6A). Comparison to GenBank sequences suggests that HEN2 is a putative DExH-box RNA helicase. HEN2 contains all 7 of the conserved DExH-box helicase motifs with almost 100% identity to the motifs predicted for the Ski2p family to which HEN2 appears to belong (Fig. 6A,B) (de la Cruz et al., 1999). The proline to leucine substitution in HEN2-1 affects a highly conserved proline immediately following helicase motif V (found in all homologs identified) (Fig. 6A,B), suggesting that the secondary structure of the protein may be disrupted, interfering with helicase activity in the mutant. The N- and C-terminal portions of the protein, however, contain no conserved motifs, with the exception of a Robbins-Dingwall nuclear localization signal in the middle of the C-terminal region predicted by PSORT (Nakai and Kanehisa, 1992).

While the N terminus of HEN2 is not conserved, both the helicase region (residues 71-525) and the C-terminal region (residues 526-991) are very highly conserved amongst proteins from various eukaryotes (up to 59% and 36% identity, respectively; up to 76% and 58% similarity, respectively) (Fig. 6B). HEN2 has three predicted paralogous proteins from *Arabidopsis* (F23H11.8, F13I12.10 and F20P5.20), with the similarity restricted to the amino acid sequence. The highest

A

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MEEPETLGKR KESESSKLRS DETPTPEPRT KRRSLKRACV HEVAVPNDYT 50
PTKEETIHGT LDNPVFNGDM AKTYPFKLDP FQSVSVACLE RKESILVSAH 100
TSAGKTAVAE YAIAMAFRDK QRVIYTSPLK ALSNQKYREL QHEFKDVGLM 150
TGDVTLSPNA SCLVMTTEIL RAMLYRGSEV LKEVAWVIFD EIHYMKDRER 200
GVVWEESIIF LPPAIKMVFL SATMSNATEF AEWICYLHKQ PCHVVYTDFR 250
PTPLQHYAFP MGGGGLYLVV DDNEQFREDS FVKMQDTFPK PKSNDGKKSA 300
NGKSGGRGAK GGGGPGDSDV YKIVKMIMER KFEPVIIFSF SRRECEQHAL 350
SMSKLDFNTD EEKEVVEQVF NNAMQCLNEE DRSLPAIELM LPLLQRGIAV 400
                                  L
HHSGLLPVIK ELVELLFQEG LVKALFATET FAMGLNMPAK TVVFTAVKKW 450
DGDSHRYIGS GEYIQMSGRA GRRGKDERGI CIIMIDEQME MNTLRDMMLG 500
KPAPLLSTFR LSYYTILNLL SRAEGQFTAE HVIRHSFHQF QHEKALPDIG 550
NKVSKLEEEA AILNASGEAE VAEYHNLQFD IAKHEKKLMS EIIRPERVLC 600
FLDTGRLVKI REGGTDWGWG VVVNVVKNSS VGTGSASSHG GGYIVDTLLH 650
CSTGFSENGA KPKPCPPRAG EKGEMHVVPV QLPLISALSR LRISVPSDLR 700
PVEARQSILL ALQELSSRFP LGFPKLHPVK DMNIQDTEIV DLVSQIEEVE 750
QKLLAHPMHK SEDDQQIKSF QRKAEVNYEI QQLKSKMRDS QLQKFRDELK 800
NRSRVLKKLG HIDADGVVQV KGRAACLIDT GDELLVTELM FNGTFNDLDH 850
HQVAALASCF IPVDKSNEQV NLRNELTKPL QQLQDSARKI AEIQHECKLE 900
IDVEEYVEST IRPFLMDVIY SWSKGASFAE IIQMTDIFEG SIIRSARRLD 950
EFLNQLRAAA EAVGESSLES KFAAASESLR RGIMFANSLY L          991
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B

		Helicase domain (aa 71-525)	C-terminal domain (aa 526-991)
At	**F23H11.8**	59 (76)	36 (54)
At	**F13I12.10**	41 (56)	35 (58)
At	**F20P5.20**	33 (49)	19 (38)
Hs	**KIAA0052**	63 (78)	40 (60)
Dm	**35Df**	61 (77)	39 (60)
Sp	**C6F12.16C**	64 (77)	35 (56)
Sc	**Dob1p**	62 (77)	34 (57)
Ce	**W08D2.7**	56 (72)	32 (53)
Hs	**SKI2W**	46 (62)	26 (46)
Sc	**Ski2p**	48 (71)	32 (55)

scores from BLAST searches with HEN2, however, were found for proteins from other species, including ones encoded by genes from humans (KIAA0052), *Drosophila* (35Df) and yeast (Dob1p [Mtr4p] from *Saccharomyces cerevisiae*) (Fig. 6B). Only two of the homologs identified have been studied: Dob1p and Ski2p from *S. cerevisiae*. While the helicase activities of Dob1p and Ski2p have not been confirmed, they have been demonstrated to be functionally associated with a complex of RNases known as the exosome, which acts in RNA processing and degradation (Mitchell et al., 1997; de la Cruz et al., 1998; Jacobs Anderson and Parker, 1998; Bousquet-Antonelli et al., 2000).

Fig. 6. HEN2 protein sequence and similarity of HEN2 to putative DExH-box helicases in diverse eukaryotes. (A) The protein sequence of HEN2. The conserved DExH motifs are marked with double underlines. The sequences were identified according to the Ski2p family consensus as defined by de la Cruz et al. (de la Cruz et al., 1999). The nuclear localization signal is marked with a single underline. The proline to leucine substitution found in the protein encoded by *hen2-1* is in bold. (B) A schematic diagram of HEN2 showing the location of the helicase motifs (black bars) and the position of the amino acid substitution in the protein encoded by *hen2-1* (asterisk). Below is a list of the homologs from various eukaryotes. The numbers represent percentage identity and similarity (in parentheses) within the respective domains. Within the actual helicase motifs, HEN2 has 95% identity (39/41 amino acids) with the consensus of the proteins listed. *At, Arabidopsis thaliana; Hs, Homo sapiens; Dm, Drosophila melanogaster; Sp, Schizosaccharomyces pombe; Sc, Saccharomyces cerevisiae; Ce, Caenorhabditis elegans*.

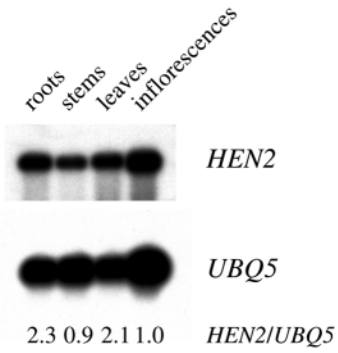

Fig. 7. RNA filter hybridization of L*er* tissues probed with *HEN2*. *UBQ5* was used to indicate the amount of RNA in each sample. The intensity of the signals was quantified with a phosphorimager and the relative abundance of *HEN2* among the samples is as indicated.

Expression pattern of *HEN2*

A *HEN2* transcript was detected in inflorescences, leaves, stems, and roots of wild-type plants by RNA filter hybridization (Fig. 7). In situ hybridization to inflorescence sections was used to determine the location and timing of *HEN2* expression in developing wild-type flowers (Fig. 8). *HEN2* mRNA was found to have a specific expression pattern both in the inflorescence meristem and in developing flowers. *HEN2* is transcribed throughout the inflorescence meristem and in young floral meristems through stage 4 (Fig. 8A-C). Around stage 5, *HEN2* RNA is lost from the sepals, but continues to be present within the developing organs of the inner three whorls (Fig. 8D-F). Late in development, the expression of *HEN2* lingers at a low level in the gynoecium and is strong in developing ovules (Fig. 8G).

DISCUSSION

HEN2 affects vegetative and inflorescence development

While *hen2-1*, *hua1-1* and *hua2-1* single mutants are wild-type in terms of plant stature, *hua1-1 hua2-1* double mutants are shorter than wild-type plants, a phenotype which is enhanced by the addition of *hen2-1*. These results suggest redundant activities of these three genes in plant growth. Such roles are

consistent with the expression of all three genes throughout the plant (Fig. 7) (Chen and Meyerowitz, 1999; Li et al., 2001). In contrast to *hua1-1* and *hua2-1*, however, *hen2-1* single mutants exhibit obvious defects in inflorescence architecture in terms of flower number and spacing both at the inflorescence tip and along the stem, as well as in flower development. This is in keeping with in situ localization data of *HEN2* transcripts, which show strong *HEN2* expression in the inflorescence meristem and developing flowers, but not in other portions of the shoot examined.

Non-homeotic role of *HEN2* in flower development

HEN2 appears to control the number and spacing of organs in the two perianth whorls. Several genes have been identified that affect floral organ number without affecting flower number or meristem size. Plants mutant for *PERIANTHA* and *ETTIN* have increased perianth organ numbers, while *tousled (tsl)* flowers show decreased numbers (Roe et al., 1993; Running et al., 1996; Sessions et al., 1997). *HEN2* appears to be unique in its effects on perianth organ number such that the *hen2-1* mutation can lead to both an increase and a decrease from that of wild type. The variable positioning, random number of organs and occasional aberrant growth direction of organs in *hen2-1* are shared with *tsl* mutants. *TSL* encodes a putative protein kinase that is needed for vegetative and ovule development in addition to floral organ initiation and placement (Roe et al., 1993).

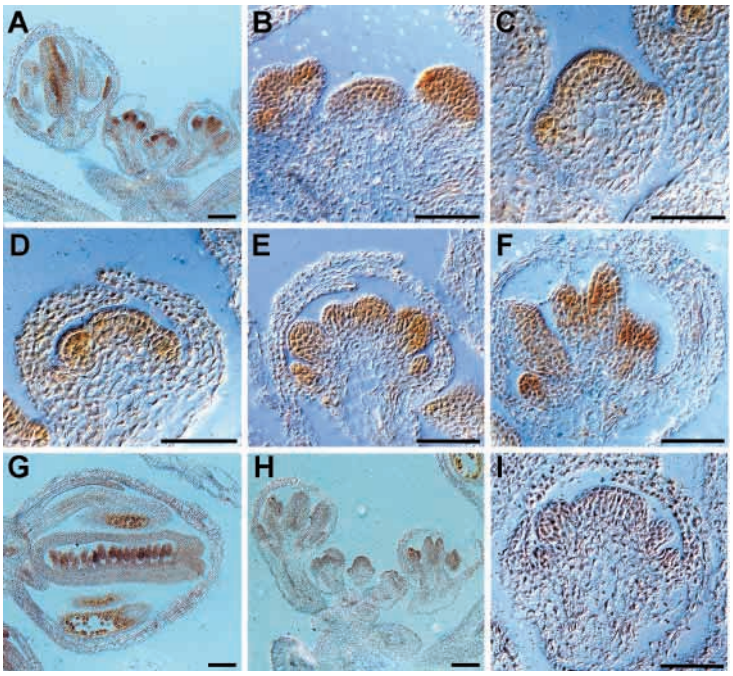

Fig. 8. In situ localization of *HEN2* RNA in L*er* inflorescences. (A-G) Antisense probe. (A) Low magnification of an L*er* inflorescence showing strong expression of *HEN2* in developing flowers of various stages. (B) An L*er* inflorescence showing expression throughout the inflorescence meristem and stage 2 and 3 floral primordia. (C) A stage 3 flower with *HEN2* expressed throughout. (D) A stage 5 flower, note *HEN2* is no longer expressed in sepals. (E) A stage 7 flower. (F) A stage 8 flower. (G) A stage 10 flower, *HEN2* is still expressed at a low level in the petals and gynoecium and strongly expressed in the developing ovules. (H,I) Hybridization with sense probe to L*er* inflorescence (H) and stage 6 flower (I), showing lack of strong signal. Scale bars, 50 μm (B-F,I), 100 μm (A,G,H).

HEN2 is required for the maintenance of B and C gene expression in developing third and fourth whorl organs

HEN2 behaves like a C function gene in that it specifies reproductive organ identity and antagonizes A function in the inner two whorls. Reproductive organ identity is compromised both in the *hen2-1 hua1-1 hua2-1* triple mutant and in double mutants with either *hua1-1* or *hua2-1*. The appearance of petal and sepal tissue in the third whorl and sepal-like cells in the fourth whorl of the triple mutant suggests the ectopic function of the A genes, *AP1*and *AP2*. The ectopic activities of *AP1* and *AP2* in the inner two whorls of the *hen2-1 hua1-1 hua2-1* triple mutant were confirmed by the finding that the perianth character in the inner two whorls was lost when either *ap1-1* or *ap2-2* was introduced into the triple mutant background. The fact that the third whorl organs of the *hen2-1 hua1-1 hua2-1 ap1-1* and *hen2-1 hua1-1 hua2-1 ap2-2* mutants are leaves instead of stamens suggests that *HEN2* plays a positive role in stamen identity specification in addition to its role in repression of A function. Since *AG* expression is reduced in mid-stages of flower development in *hen2-1 hua1-1 hua2-1* but not *hua1-1 hua2-1* and *AG* is known to specify reproductive organ identity as well as antagonize A function, it is likely that *HEN2* behaves as a C function gene by maintaining *AG* expression.

A novel aspect of the *hen2-1 hua1-1 hua2-1* phenotype is the appearance of sepal-like cells in the third whorl organs. Sepal fate is specified by the sole presence of A genes (Bowman et al., 1991a), thus it appears that not only is *HEN2* required for maintenance of C gene expression in the reproductive whorls, but also for that of B genes in the third whorl. This hypothesis was borne out both by observation of quadruple mutants with A genes and by the reduction and/or loss of *AP3* and *PI* expression from portions of third whorl organs, but not from the second whorl organs, in *hen2-1 hua1-1 hua2-1* triple mutants. Maintenance of high levels of the B transcripts occurs through auto-stimulation by an AP3-PI transcription factor complex acting directly at the promoter of *AP3* and indirectly on *PI* (Jack et al., 1994; Hill et al., 1998; Tilly et al., 1998; Chen et al., 2000; Honma and Goto, 2000). Localization of AP3 and PI proteins through immunolabelling in *hen2-1 hua1-1 hua2-1* (T. L. W. and X. C., unpublished data), however, shows a similar pattern of protein expression as seen for the mRNAs, suggesting that the autoregulation may not be compromised.

These results place *HEN2* in a new group of genes that affects the activity of multiple homeotic genes within a specific area in the developing flower. The recently defined triad of *SEP* genes affects multiple homeotic gene activity in the innermost three whorls of the flower (Pelaz et al., 2000). Like the *SEP* genes, *HEN2* is expressed in this region of the flower during the majority of flower development, but its effects on homeotic gene activity appear to be restricted to the reproductive whorls. *HEN2* also differs from the *SEP* genes in that it appears to maintain B and C gene expression, while the SEP proteins act

in complexes along with their fellow MADS box proteins, AP1, AP3, PI and AG, and serve as the determinants for floral organs on which the homeotic genes confer identity (Honma and Goto, 2001; Pelaz et al., 2001a; Pelaz et al., 2001b). *HEN2* does not appear to be involved in the regulation of *SEP* gene expression as all three SEP genes are expressed normally in the *hen2-1 hua1-1 hua2-1* triple mutant.

The reproductive-to-perianth organ transformation in *hen2-1 hua1-1 hua2-1*flowers is much more severe than that in *hen2-1 hua1-1* or *hen2-1 hua2-1* flowers. Therefore, both *hua2-1* and *hua1-1* affect C function in the inner two whorls and B function in the third whorl. It is likely that both *HUA1* and *HUA2*, like *HEN2*, act to maintain the expression of the B and C genes in the inner two whorls, although this has to be tested by comparing the expression of B and C genes in the triple with the above-mentioned double mutants.

Early versus late roles of B and C genes during flower development

In *hen2-1 hua1-1 hua2-1* flowers, the expression of the floral homeotic genes *AP1, AP3, PI* and *AG* is correctly established and continued into the appropriate organ primordia. It is only about stage 6 that the expression of those genes in the reproductive whorls becomes patchy, correlating with the mosaicism seen in the mature organs. These results emphasize the presence of distinct phases in B and C gene expression: initial establishment of expression in organ primordia followed by maintenance throughout the organ primordia as they commence differentiation. This is consistent with previous findings that distinct mechanisms regulate the initiation and the maintenance of B gene expression (Jack et al., 1992; Goto and Meyerowitz, 1994; Parcy et al., 1998; Homna and Goto, 2000). In addition, our results demonstrate the significance of the maintenance phase of homeotic gene expression. The organ identity defect in the inner two whorls of *hen2-1 hua1-1 hua2-1* flowers suggests that sustained expression of B and C genes is required for organ identity. This is in agreement with early temperature shift studies done with the weak *ap3-1* allele (Bowman et al., 1989) where it was shown that *AP3* activity is necessary until just before third whorl organ differentiation becomes visible. Our studies also suggest that the restriction of A activity and/or expression by *AG* needs to be maintained into the differentiation phase of flower development.

Another intriguing aspect of the phenotype of *hen2-1 hua1-1 hua2-1* is its determinacy in the face of reduced *AG* levels. Studies of the weak *ag-4* allele plus strains with reduced *AG* levels have suggested that, of the three *AG* functions (reproductive organ identity, repression of A genes and determinacy), determinacy is the function that requires the highest level of *AG* activity (Mizukami and Ma, 1995; Sieburth et al., 1995). This is the opposite of what is seen in *hen2-1 hua1-1 hua2-1*. This result suggests that determinacy (or at least the requirement for high levels of *AG*) is concluded prior to stage 6 in flower development. This is in agreement with the activity of *AG* as a repressor of *WUS* in the floral meristem, whose expression is lost prior to stage 6 (Lenhard et al., 2001; Lohmann et al., 2001).

HEN2 belongs to a family of highly conserved putative DExH-box RNA helicases

HEN2 appears to belong to a family of very highly conserved proteins found in diverse eukaryotes. The family members share a divergent N terminus, a well-conserved helicase domain, including the seven DExH helicase motifs, and a C-terminal region unique to this group of proteins. DExH-box helicases make up a sub-group of the DEAD-box helicases that compose the majority of helicase superfamily II (de la Cruz et al., 1999). While several DEAD-box helicases, including AtDRH1 from *Arabidopsis* (Okanami et al., 1998), have been demonstrated to have double-stranded RNA unwinding activity, the majority of the family members have been identified through sequence homology (de la Cruz et al., 1999). A large number of DEAD-box family helicases have been identified in *Arabidopsis* (Wei et al., 1997; Okanami et al., 1998; Auborg et al., 1999; Isono et al., 1999), but a mutant has been described only for *CARPEL FACTORY (CAF)* (Jacobsen et al., 1999). *CAF* encodes a protein that contains a helicase domain from the DEAH sub-group along with an RNaseIII-like domain. The role of *CAF* in flower development is quite different from *HEN2*: *caf* flowers are indeterminant with increased numbers of stamens and carpels without homeotic changes. HEN2 is the first DExH-box helicase of the Ski2p sub-group to be described in plants. *HEN2* has three paralogs in the *Arabidopsis* genome.

The only HEN2 homologs of known function are the *S. cerevisiae* proteins Dob1p (Mtr4p) and Ski2p. Both helicases are functionally associated with an RNase complex, the exosome, with Dob1p acting in the nucleus and Ski2p in the cytoplasm (Mitchell et al., 1997; de la Cruz et al., 1998; Jacobs Anderson and Parker, 1998). Both higher sequence similarity and the presence of a nuclear localization signal in HEN2 suggest that it is more likely to be an ortholog of Dob1p than Ski2p. While one component of the exosome from *Arabidopsis* has been described (Chekanova et al., 2000), HEN2 is the first component for which a mutant allele has been isolated. The nuclear exosome, including Dob1p, was first implicated in the processing of rRNA and thus ribsome biogenesis (de la Cruz et al., 1998). More recently, however, it has also been shown to act in the 3′ to 5′ degradation of pre-mRNA, thus acting more directly in the regulation of gene expression (Bousquet-Antonelli et al., 2000).

Action of HEN2 in the regulation of gene expression

How could HEN2, a potential nuclear RNA helicase, maintain the level of *AP3, PI* and *AG* mRNAs during the mid-stages of flower development? One possibility is that HEN2 acts through the exosome that functions in ribosome biogenesis and mRNA degradation. In this scenario, the effect of *hen2-1* on homeotic gene expression is not specific. *hen2-1* may lead to reduced ribosome numbers or defects in mRNA degradation. Homeotic gene expression could be affected by the stochastic loss of their maintenance factors. Alternatively, HEN2 may not act as an exosome-associated helicase. One of the HEN2 paralogs in *Arabidopsis*, F23H11.8, has a higher identity to Dob1p, suggesting that it may be a better candidate for an exosome role.

RNA helicases have been shown to act in diverse processes in gene expression, such as transcription, RNA splicing, RNA transport, RNA stability, and translation (de la Cruz et al., 1999). HEN2, therefore, may directly or indirectly regulate homeotic gene expression in one of these processes. Although RNA helicases generally do not recognize substrates by

sequence, mutations in many RNA helicases lead to specific developmental defects (Hay et al., 1988; Kuroda et al., 1991; Jacobsen et al., 1999; Puoti and Kimble, 1999; Kuznicki et al., 2000; Schmucker et al., 2000; Navarro et al., 2001), suggesting that some sets of RNAs are more sensitive than others to alterations in RNA helicase function. Such specificity may derive in part from RNA-binding proteins that interact with the substrates. HUA1, a CCCH zinc finger protein that has been shown to bind RNA (Li et al., 2001), may confer substrate specificity to HEN2.

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